*LAWSONIA INTRACELLULARIS*

DISEASE CONTROL AND EPIDEMIOLOGY


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**Dedication**


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**Abstract**

Proliferative enteropathy (PE) is an important enteric disease in grower and finisher pigs caused by an obligate intracellular bacterium, *Lawsonia intracellularis*. The overall aims of this thesis were to obtain more information about the *in vitro* activity of antimicrobials and disinfectants against *L. intracellularis* and to develop a new quantitative PCR assay.

To determine the minimum inhibitory concentration (MIC) of antimicrobials against *L. intracellularis*, ten isolates obtained from North America and Europe were tested against 6 antimicrobials using a modified tissue culture assay. *In vitro* results found that carbadox, tiamulin, and valnemulin were the most active antimicrobials, chlortetracycline and tylosin were intermediately active, lincomycin was the least active against *L. intracellularis*, and the antimicrobial sensitivity patterns differed across isolates.

We next evaluated the effectiveness of the modified tissue culture and direct count method with special fluorescence to determine *in vitro* disinfectant activity against *L. intracellularis*. The outcomes of both methods predicted similar *in vitro* bactericidal activities against *L. intracellularis* with a high degree of correspondence. This suggests that either assay would be appropriate in determining the bactericidal activity of disinfectants against *L. intracellularis*. Based on our *in vitro* results, we predict that a powder disinfectant (Stalosan® F), DC&R®, Roccal®-D, Synergize®, and Virkon®-S would perform well under field conditions, while Certi-Dine®, Nolvasan®-S, and Tek-Trol® would be less active against *L. intracellularis*.

Finally, a new quantitative PCR assay was developed using a SYBR green system. The assay showed negative results when DNA from 16 other species of enteric bacteria as well as 20 negative control samples of pig feces was tested. Quantitative estimates from the assay were roughly 2-fold lower than the expected values across all dilutions of pure culture and spiked feces. Validation results indicated that this new qPCR is sensitive, specific, reliable, accurate, and precise for the detection and quantification of *L. intracellularis* in different types of samples.

In conclusion, the results of these investigations update and expand upon existing information about the *in vitro* antimicrobial and disinfectant activities against *L. intracellularis.* Further, the development of a new, accurate, and precise qPCR assay will facilitate future epidemiology studies.

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**GENERAL INTRODUCTION**

Proliferative enteropathy (PE), or ileitis, was first recognized in the North American swine industry in the early 1930s. Since then, this disease has become a major enteric health concern for swine production in the U.S. and around the world. After the first pure bacterial culture of *Lawsonia intracellularis* was successfully isolated and maintained *in vitro* in 1993 (Lawson et al., 1993), an increasing number of investigations were conducted in order to understand the nature of the bacteria and find the ways to cure and prevent the disease. However, several areas of research require further understanding and investigation.

When an outbreak of PE occurs in a herd, several strategies of treatment and control may be used to stop the progression of disease and to prevent future outbreaks. Antimicrobial therapy is usually the first preference because it is able to control a PE outbreak in a short period of time. Therefore, antimicrobial selection is critical for achieving the best possible outcome. In general, the treated antimicrobials are usually selected based on the *in vitro* sensitivity of the causative agent. However, the isolation of *L. intracellularis* from infected intestine or fecal samples is extremely difficult and the process of isolation requires several months to establish a pure culture. Consequently, *in vitro* sensitivity results are nearly impossible to obtain in time to treat an outbreak. Furthermore, the obligate intracellular nature of *L. intracellularis* prohibits the use of standard antimicrobial susceptibility methods. Instead, a complicated tissue culture system has been used to evaluate antimicrobial activity against a few isolates of *L. intracellularis* (McOrist et al., 1995c). In order to expand the limited information on *in vitro* antimicrobial sensitivity, additional primary isolates of *L. intracellularis* must

be isolated and tested. Further, a new, reliable, and easy-to-perform *in vitro* sensitivity assay needs to be developed for use with *L. intracellularis*.

Disinfectant application is the most effective strategy for controlling *L. intracellularis* in the environment. However, little is known about the bactericidal activity of disinfectants against *L. intracellularis*. Therefore, it is difficult to identify the most effective disinfectants among products that are commercially available. Only one *in vitro* study has been reported in which the bactericidal activity of some disinfectants was measured using a conventional tissue culture assay (Collins et al., 2000). However, this assay had several limitations, including time-consuming procedures, and the inability to distinguish between viable and non-viable bacteria after exposure to disinfectants. Further investigation into a new method for determining the bactericidal activity of various disinfectants against *L. intracellularis* is still required. Once a new assay is established and validated, it can be applied to future disinfectant evaluations to permit the selection of those that are most effective in the control of the disease. Unfortunately, there are also no scientific reports on the validation of disinfectant efficacies against *L. intracellularis* under field conditions.

Little is known about the dynamics and quantity of *L. intracellularis* excretion through feces after both experimental and natural infection. These limitations are due to the lack of an assay that accurately quantifies *L. intracellularis* in fecal samples. Conventional PCR can only be used to detect the presence or absence of *L. intracellularis* DNA in fecal samples, not the quantity. Not only is the quantity of *L. intracellularis* in fecal samples difficult to estimate, but the number of bacteria in pure culture or mucosal homogenate inoculum are also difficult to assess. To date,

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estimations of *L. intracellularis* in such samples have been conducted with an immunostaining assay with an antibody specific to *L. intracellularis*. Additional limitations of immunostaining assays include the time-consuming process, subjective results, and the fact that only a few laboratories can perform the test in the absence of commercially available monoclonal or polyclonal antibodies specific for *L. intracellularis*. Therefore, simple, reliable, accurate, and precise assays to quantify *L. intracellularis* in fecal, pure culture, and tissue samples are required.

The overall aim of these investigations was to obtain more information about the *in vitro* susceptibility of *L. intracellularis* to antimicrobials and the *in vitro* bactericidal activity of disinfectants against *L. intracellularis* for use in the treatment and control of PE. Additionally, our investigations seek to develop a new quantitative assay for the accurate and precise quantification of *L. intracellularis* in samples. This assay can be used in future studies to generate epidemiological information about the dynamics of disease progression in experimental circumstances and natural PE outbreaks.

This thesis is composed of 4 sections: Section I is a general introduction and literature review. Sections II and III are research studies conducted in order to achieve the overall goals. Section IV is the conclusion of these investigations. Section I, chapter I is a literature review on the basic knowledge of *L. intracellularis*, the causative agent of proliferative enteropathy in swine. It also includes remarks about the need for further investigation. Section II contains chapters 2 through 4, which describe the *in vitro* activity of antimicrobials and disinfectants against *L. intracellularis*. Studies in chapter 2 entail the *in vitro* activity of 6 antimicrobial agents commonly used for the treatment and control of PE in pigs against 10 *L. intracellularis* isolates obtained from the United

States and Europe. Several new primary cultures of *L. intracellularis* were successfully isolated and a new modified tissue culture system was developed to assess the minimum inhibition concentrations (MICs) of antimicrobials against those isolates of *L. intracellularis*. Studies in chapter 3 describe the use of modified tissue culture assay compared to the direct count method stained with specific fluorescence for measuring the effectiveness of a powder disinfectant (Stalosan® F) against *L. intracellularis*. Chapter 4 describes a study in which a modified tissue culture was used to measure the bactericidal activity of 7 commercial disinfectants commonly used in swine facilities under conditions of synthetic hard water and organic material load. Section III, chapter 5 describes the development and validation of a new quantitative polymerase chain reaction (qPCR) for the direct quantification of *L. intracellularis* in swine feces and pure culture. The new qPCR was validated with several sets of spiked fecal samples and serial 10-fold dilutions of pure culture bacteria in terms of accuracy and precision of bacterial quantity estimations. Section IV contains a summary and conclusion of the research outcomes. The results of this thesis will hopefully be useful to veterinarians for the treatment and control of PE as well as for future epidemiological studies.

# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction

Proliferative enteropathy (PE), or ileitis, is an important infectious enteric disease in grower and finisher pigs. The disease is caused by the intracellular bacterium *Lawsonia intracellularis* and is characterized by thickening of the intestinal mucosa at the distal part of the small intestine. In cases of severe infection, the proliferative lesions can sometimes extend to the proximal part of the small intestine or the large intestine (Lawson and Gebhart, 2000).

Ileitis in pigs has three clinical presentations. An acute form of ileitis, called porcine hemorrhagic enteropathy (PHE), is clinically observed as bloody diarrhea and leads to sudden death in adult pigs. The chronic form of ileitis, called porcine intestinal adenomatosis (PIA), is usually found in younger pigs and is characterized by poor growth and chronic diarrhea due to abnormal proliferation of the small intestinal mucosa. The subclinical form of ileitis presents as slow growth with no outward signs of diarrhea (McOrist and Gebhart, 2006).

## 1.2 Etiology

The first clinical case of PE in pigs was reported in North America in 1931 (Biester and Schwarte, 1931). However, the etiological agent of PE remained unidentified for many years. Forty or more years later, curved, intracellular bacteria were first described in the cells of an abnormal pig intestine (Rowland et al., 1973), and the bacteria were observed and identified using conjugated immunofluorescence and electron microscopy (Rowland and Lawson, 1974). This intracellular organism was previously known as a *Campylobacter*-like organism (CLO), *Ileal symbiont*

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*intracellularis* and *Ileobacter intracellularis*. It was found that the unique characteristics of the intracellular organism prevented its ability to be cultured with standard media and required a specific atmosphere for microbial growth.

After 60 years of mystery since the first case report, the intracellular bacteria were ultimately successfully isolated and identified via tissue culture system growth under a specific atmosphere (Lawson et al., 1993). The newly identified obligate intracellular bacteria were classified with the molecular taxonomic procedure of 16S rDNA sequence analysis. Using this molecular approach, the bacterial sequence was 91% similar to *Desulfovibrio desulfuricans* (Gebhart et al., 1993) and 92% related to a free-living anaerobic human pathogenic organism named *Bilophila wadsworthia* (Sapico et al., 1994). The PE lesion in pigs was experimentally reproduced by inoculating pigs with a pure culture of bacteria to fulfill Koch's postulates and to confirm that this organism is the causative agent of PE (McOrist et al., 1993). Finally, the new genus and species name of this organism was established in the family *Desulfovibrionacae* and named *Lawsonia intracellularis* to honor Dr. Lawson GHK for his contribution to the landmark discovery of PE's causative agent *(*McOrist et al., 1995a).

*L. intracellularis* is a Gram-negative, curved or sigmoid-shaped bacterium, approximately 1.25 to 1.75 μm in length by 0.25 to 0.43 μm in width. The phenotypic structure of *L. intracellularis* includes a long, single, uni-polar flagellum but no fimbriae or spores. The membrane wall of *L. intracellularis* contains a tri-laminar outer envelope which is normally divided from the cytoplasmic membrane by an electron-

lucent zone (Lawson and Gebhart, 2000). The genome of *L. intracellularis* contains a single small circular genome and three plasmids, approximately 1.72 million bp and 1,324 open reading frames (ORF) (McOrist and Gebhart, 2006).

**1.3 Pathogenesis**

The pathogenesis of *L. intracellularis* infection in pigs is not fully understood. However, since the pure culture of *L. intracellularis* was successfully isolated and maintained under *in vitro* conditions (Lawson et al., 1993), several *in vivo* and *in vitro* studies have been conducted to understand the pathogenic mechanisms. Using rat (IEC-18) and pig enterocyte (IPEC-J2) tissue cultures as models, *L. intracellularis* was observed to attach to the eukaryotic cell membrane within 10 min post inoculation (McOrist et al., 1995b). Later, the bacteria entered into the enterocytes via membrane-bound vacuoles and were released into the cell cytoplasm with vacuole breakdown. Several studies show that the invasion of bacteria into the enterocytes occurs within 3 hours of bacterial inoculation (Lawson et al., 1993; McOrist et al., 1995b). Interestingly, this invasion does not depend on bacterial viability. Lawson et al. (1995) showed that both live and formalin-killed bacteria could penetrate into enterocytes, and this internalization was dramatically reduced at 20$^{o}$C. Furthermore, when the metabolism of enterocytes was reduced by chilling the cell to 5$^{o}$C or adding cytochalasin D, which is an inhibitor of actin polymerization, neither live nor killed bacteria could enter and proliferate within the cells. This suggests that host cell function and metabolism have an effect on the bacterial internalization mechanism. Once the bacteria were internalized in the cell, they multiplied freely in the cytoplasm of the monolayer cells with peak

numbers of infected bacteria occurring by day 6 post-infection. Bacteria were then released to the outside through cytoplasmic protrusions (McOrist et al., 1995b).

The mechanisms of *L. intracellularis'* entery and escape from the enterocyte in a tissue culture model are similar to the results of the *in vivo* model. In pigs challenged with pure culture *L. intracellularis* or infected intestinal mucosal homogenate, the bacteria were not shed in feces before 6 to 7 days post-infection (Smith and McOrist, 1997). The bacteria could be detected in feces using the polymerase chain reaction (PCR) technique as early as 7 days after oral inoculation (Knittel et al., 1998; Guedes and Gebhart, 2003b). However, Collins and Love (2007) demonstrated that the onset of *L. intracellularis* fecal shedding could be delayed to 19 days post-inoculation with low doses of *L. intracellularis* inoculum ($10^5$ *L. intracellularis* organisms). In contrast, the excretion of *L. intracellularis* through feces was found to be as fast as 7 days post-inoculation with high doses of inoculum ($10^{10}$ *L. intracellularis* organisms). These findings indicate that the onset of fecal shedding is also dependent on the infectious dose of the inoculum.

In challenged animals, the peak of infection was found to be 3 weeks after challenge, with 50% of challenged animals showing moderate diarrhea and 100% exhibiting histological lesions (Guedes and Gebhart, 2003b). The proliferative lesions were found 3 weeks after infection and occurred mainly in the ileum but were sometimes extended to the jejunum or colon (Guedes and Gebhart, 2003b). The proliferative lesions and bacterial excretion from the infected pigs persisted for approximately 4 to 6 weeks (Collins and Love, 2007). However, the fecal shedding of

*L. intracellularis* has been observed for up to 10-12 weeks post-inoculation (Smith and McOrist, 1997; Guedes and Gebhart, 2003b).

**1.4 Epidemiology**

PE in pigs has been reported throughout the United States and worldwide. Based on fecal sample PCR detection, estimates of herd infectivity rates with *L. intracellularis* range from 4.5 to 50% in the U.S., Brazil, Denmark, Korea, and Taiwan; PE within-herd prevalence estimates range from 3.3 to 30% (Winkelman, 1996a; Chang et al., 1997; Kim et al., 1998; Chiriboga et al., 1999; Stege et al., 2001; Marsteller et al., 2003). Positive fecal PCR results are indicative of disease presence-either currently or in the recovering stages.

In the U.S., a mass serological survey by the National Animal Health Monitoring System (NAHMS) found that nearly 100% of swine herds were seropositive for *L. intracellularis* infection (Bane et al., 1997). Additionally, a recent study of *L. intracellularis* seroprevalence from 174 separate pig flows across the U.S. during the years 2003 to 2006 reported that 91% of flows were seropositive for at least one age in a cross-sectional blood collection (Armbruster et al., 2007). Positive serology results indicate pigs which may no longer have the disease but had prior exposure to the bacteria. The high rates of seroprevalence indicate that most pigs are exposed to *L. intracellularis* in the herd or environment at least once in the production system.

Several studies have been conducted to identify risk factors associated with PE infection. Stage et al. (2001) reported that pigs in continuous production systems using commercial feed products have a higher risk of *L. intracellularis* infection as compared

to batch production using home-mixed feed. A case-control study conducted in the U.S. reported that new facilities (less than 1 year old) and pigs mixed from different sources are more likely to have a PE outbreak (Bane et al., 2001). Furthermore, pigs from large herds have an increased risk towards PE occurrence relative to those of small herds (Holyoake et al., 1994a; Smith et al., 1998).

Bronsvoort et al. (2001) described that risk factors associated with PE outbreaks in breeding herds include seropositivity to *L. intracellularis* during the growing-finishing phase, a continuous flow system for the farrowing unit, and younger sow parity. On the other hand, risk factors for a PE outbreak in a growing-finishing herd include seropositive status of the breeding unit, a high number of pigs entering the facilities, the use of concrete slats as flooring, and intensive indoor management.

Porcine PE has been reported in all types of pig farm management systems, including those raising pigs outdoors (Class and Bilkei, 2004). Interestingly, when the seroprevalence of grower-finisher pigs raised in indoor facilities was compared to that of pigs raised in outdoor facilities, the indoor pigs showed higher number of seropositive with slowly turn to be negative pigs relative to those raised in outdoor facilities. This suggested that re-exposure rates among pigs raised in indoor facilities are higher than those of pigs raised outdoors (Bona and Bilkei, 2003; Class and Bilkei, 2004).

Pig flows and management systems are important factors that have changed the dynamics of within-herd disease spread. The dynamics of disease spread differ between single- and multi-site productions. In single-site systems with continuous pig flow (called farrow-to-finish), seroconversion was found early in the growing phase of pigs 8

to 16 weeks of age. This indicates that the infection may occur at approximately 5 to 7 weeks of age— after passive maternal immunity disappears. The clinical signs in these flow systems have often been associated with subclinical disease and PIA (Chouet et al., 2003; Stege et al., 2004). On the other hand, in multi-site systems where pigs were transferred across several locations according to growth phase, the disease rarely occurred in breeding herds but the seroconversion was usually detected late in the finisher period among pigs 16 to 20 weeks of age (Just et al., 2001; Chouet et al., 2003) or 12 to 21 weeks of age (Lasley et al., 2009). Moreover, there were no significant differences in the rate of seroconversion to *L. intracellularis* in the barns with partially slatted floors versus fully slatted floors in a multi-site production system (Lasley et al., 2009). The clinical signs of the older pigs' infections were often associated with the PHE or PIA form.

*L. intracellularis* is transmitted from pig to pig through a fecal-oral route (McOrist and Lawson, 1987). It has been confirmed that the bacteria can survive outside the host and remain infective for at least 2 weeks (Collins et al., 2000). Therefore, fecal material contaminated with *L. intracellularis* may be an important source of horizontal disease transmission. Little is known about the post-infection dynamics of fecal bacterial excretion. Several investigations have reported that bacterial excretion in infected pigs occurs 1 to 3 weeks after infection (Knittel et al., 1998; Guedes and Gebhart, 2003ab; Collins and Love, 2007) and infected pigs can continuously shed the organism through feces for up to 10 to 12 weeks (Smith and McOrist, 1997; Guedes et al., 2002a). However, little is known about when bacterial excretion peaks and how much bacteria are excreted after infection. One experimental

study estimated the number of organisms shed from infected pigs to be approximately $5 \times 10^4$ to $7 \times 10^8$ *L. intracellularis/*gram of feces (Smith and McOrist, 1997). However, this study neither reported how much bacteria was excreted at each time point across the infection nor when the peak of excretion occurred.

The estimation of bacterial excretion from the previous study was performed using an immunofluorescence assay with a serial dilution of fecal material. The bright green, curved, fluorescencing bacteria were then counted under a fluorescence microscope. The limitations of this assay included subjective evaluation of results, heavy time consuming, and lower diagnostic specificity. Additionally, only a few laboratories around the world can quantify the amount of bacteria in samples because the assay relies on specific antibodies. Therefore, the development of a new, reliable, and objective method to quantify bacteria levels in feces is essential.

In endemic herds with naturally occurring PE outbreaks, the fecal shedding of *L. intracellularis* was initially observed in pigs 10 to 12 weeks of age (approximately 22-29 kg), and infected pigs shed the bacteria continuously for 2 to 6 weeks. PCR was unable to detect the number of bacteria in feces after 18 weeks of age. Seroconversion among infected pigs was first detected at 12 to 14 weeks of age. Seroconversion was delayed 1 to 2 weeks after the fecal shedding was first detected and remained positive until 20 to 24 weeks of age (Stege et al., 2004). However, the earliest reports of natural *L. intracellularis* fecal shedding were in piglets 3 weeks old (López et al., 2000), at 10 to 24 days after weaning (Møller et al., 1998), and at 7 weeks of age (Guedes et al., 2002a). Since fecal shedding has been detected in the early nursery phase, it is possible that infection occurred during the lactation period and the sow was a source of

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transmission. However, this vertical transmission hypothesis from sow to piglet has not yet been confirmed due to a lack of methods for identifying bacterial strain variations across sows and their piglets.

To date, strains of *L. intracellularis* can be differentiated and identified using an advanced molecular technique called variable number tandem repeat (VNTR) sequence. This molecular assay has been primarily used to identify strain variation among *L. intracellularis* isolates from natural PE outbreaks within herds (Beckler et al., 2004). Molecular genetic typing will have a great impact on future molecular epidemiology studies. For instance, it can be used for tracking the movement of bacteria within and between herds, studying strain variation of bacteria originating from pigs and other species, and identifying the sources of PE infection.

To date, the primary source of natural PE outbreaks that occur in the field remains unclear. Inter-species transmission of *L. intracellularis* in the farm from small mammal species to pigs may be possible since *L. intracellularis* infection has been reported in several small rodent species (McOrist and Lawson, 1987; Vandenberghe and Marsboom, 1982; Abshier et al., 2001). However, these hypotheses have not yet been confirmed. Recently, several studies found that some small rodent species and small terrestrial mammals free-living in close proximity to swine and horse farms were positive to *L. intracellularis* infection with both PCR and serology (Friedman et al., 2008; Pusterla et al., 2008). Although positive results were obtained from these animals, it has not been determined if they were the primary source of infection or merely received the bacteria from the infected pigs and horses.

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Further studies are required to investigate the dynamics of disease distribution within the herd, to develop a new quantitative assay for quantification of *L. intracellularis* in feces, to understand the role of vertical and vector transmission, and to identify the primary source of disease outbreaks.

**1.5 Diagnosis**

The primary isolation of *L. intracellularis* from feces or infected intestine is both difficult and complicated. The cultivation procedure requires an expensive incubator in order to set up a specific atmosphere, and establishing a pure culture requires several months (Lawson et al., 1993). Less than 20 *L. intracellularis* isolates have been successfully cultured and maintained worldwide. Therefore, the diagnosis of PE infection through bacterial culture from fecal material or infected intestine is not a practical choice for routine diagnosis.

In postmortem diagnosis, PE infection in pigs is usually confirmed by staining histopathologic lesions with hematoxylin and eosin (H&E), Warthin-Starry silver stains, or an immunohistochemistry technique (Lawson and Gebhart, 2000). However, these post-mortem tests have limitations with respect to sensitivity of bacterial detection. Sensitivities of H&E and Warthin-Starry silver staining are reported to be 36.8% and 50%, respectively, while the immunohistochemistry staining technique has a reported sensitivity of 86.8% (Guedes et al., 2002d). In fact, the intracellular organisms within the lesions can be seen only with silver stains and monoclonal-antibody-specific immunohistochemistry (Guedes et al., 2002d; Van der Heijden et al., 2004). Estimates of PE prevalence through postmortem tests of slaughter-age pigs are prone to

underestimation because most lesions in the infected pigs have healed by slaughter time (Holyoake et al., 1994a; Van der Heijden et al., 2004). Van der Heijden et al. (2004) have suggested that the use of antemortem tests such as PCR or serology provide more accurate estimates of PE prevalence than do postmortem examinations of gross lesions at slaughter.

There are currently several diagnostic tests available for antemortem diagnosis. PCR and immunoperoxidase assays are used to detect antigen in the samples while serological assays are used to detect specific antibodies against *L. intracellularis* infection. Advantages of antemortem tests over postmortem tests include the ability to monitor infection status in live animals and more accurate estimation of disease prevalence.

For antigen detection, the presence or absence of *L. intracellularis* in feces can be determined using an indirect antibody staining technique and a PCR assay. The use of indirect antibody staining for the detection of *L. intracellularis* in feces has been reported in several studies (Smith and McOrist, 1997; Guedes et al., 2002d). However, this assay requires a high-quality of *L. intracellularis*-specific antibody which is not available worldwide, and the test's outcome is subjective and requires well-trained people for interpretation. Additionally, this assay is limited by its sensitivity for detection of low concentrations of *L. intracellularis* in fecal samples (Guedes et al., 2002d).

The PCR assay is the best antemortem diagnostic test to detect the presence or absence of *L. intracellularis* DNA in feces and tissue. Several studies have used PCR to monitor the dynamics of disease in both experimental animals and natural disease

outbreaks (Jones et al., 1993ab; Knittel et al., 1998; Guedes et al., 2002ad; Jacobson et al., 2004). Although fecal PCR is widely used for DNA detection, its sensitivity only ranges between 40% and 70% due to the effects of fecal inhibitors (Knittel et al., 1998; Guedes et al., 2002d). The lowest quantity of *L. intracellularis* in feces that conventional PCR has been reported to detect is $10^3$ *L. intracellularis/*g of feces (Jones et al., 1993b); however, nested PCR is able to detect bacterial concentrations as low as $2x10^2$ *L. intracellularis*/g of feces. Conventional PCR is a qualitative assay that detects only the presence or absence of bacterial DNA in samples. Therefore, it is unable to assess the quantity of *L. intracellularis* in those tested samples. A new quantitative assay to accurately and precisely estimate the number of bacteria in feces and other samples needs to be developed.

Real-time or quantitative PCR (qPCR), an advanced molecular assay, has been widely used for the detection and quantification of organisms in samples. Two qPCR assays have been previously developed with the main purpose of *L. intracellularis* detection in porcine and equine fecal samples (Lindecrona et al., 2002; Pusterla et al., 2008). Aother recent study developed a new qPCR for the purpose of *L. intracellularis* quantification in swine feces (Goy et al., 2008; Nathues et al., 2009). However, each of these studies has developed qPCRs using probe-based systems which are highly specific but more expensive and less sensitive. Therefore, a new qPCR assay that provides a cheaper cost/high throughput option with higher sensitivity needs to be developed and validated.

Several serological tests are available for antibody detection, including indirect fluorescent antibody tests (IFATs), enzyme-linked immunosorbant assays (ELISAs),

and an immunoperoxidase monolayer assay (IPMA). The sensitivity for determining exposure status of pigs of the serological tests is better than that of the fecal PCR assay (Guedes et al., 2002bcd; Knittel et al., 1998; Huerta et al., 2003) and fecal immunoperoxidase (IPX) (Guedes et al., 2002d). Therefore, serological assays are widely used to estimate the seroprevalence of PE in the field (Just et al., 2001; Guedes et al., 2002a; Class and Bilkei, 2004; Stege et al., 2004).

All current serological tests measure the immunoglobulin G (IgG) response in the serum. The importance of serum IgG has remained unclear but it provides useful information for estimating seroprevalence and the approximate time of infection. An IgG response against *L. intracellularis* infection develops within 14 to 21 days after exposure (Knittel et al., 1998; Guedes et al., 2002bc) and remains detectable in the serum for as long as 4 (Just et al., 2001) to 12 weeks post infection (Guedes et al., 2003b). Therefore, a series of cross-sectional or longitudinal blood collections can be used to estimate the time of exposure and disease prevalence within herds.

The IFAT was the first serological test developed to detect antibodies specific to *L. intracellularis* in pigs (Lawson et al., 1988). At that time, the assay used antigen obtained directly from the intestinal mucosa of infected pigs. It could detect serum immunoglobulin (Ig) M and IgA but it failed to detect an IgG response (Lawson et al., 1988). After the pure culture of *L. intracellularis* was successfully isolated, the IFAT was developed using a combination of pure culture and eukaryotic cells as antigen, and the IgG response was successfully detected within 3 weeks after infection (Knittel et al., 1998). Later, the tissue-culture IFAT was modified to use only pure *L. intracellularis* as antigen (without eukaryotic cells) to reduce the background fluorescence. Both IFAT

assays have the same 91.2% sensitivity with 94.4% agreement on the test outcome (Guedes et al., 2002d). The specificity of the IFAT has been estimated to be as high as 97% (Huerta et al., 2003).

The IPMA was developed later for the serological diagnosis of PE. Its sensitivity is similar to the IFAT (88.9%), but it shows perfect specificity (100%). Furthermore, agreement between the IFAT and IPMA outcomes has been reported as high as 98.6% (Guedes et al., 2002c). Like the IFAT, IPMA results are subject to reader interpretation, but the major advantage of IPMA over IFAT is that it does not require an expensive fluorescent microscope. Moreover, the red color of the IPMA staining remains stable for several months while the fluorescent color of the IFAT remains stable for just a few hours due to deterioration of the fluorescent-tagged antibodies (Guedes et al., 2002bc).

Several ELISA systems have been developed for the objective interpretation of PE serology results (Holyoake et al., 1994b; Keller et al., 2004; Boesen et al., 2005; Kroll et al., 2005; Wattanaphansak et al., 2008). The first ELISA was developed using unpurified antigen obtained directly from affected intestinal mucosa. Although the test could detect an IgG response in natural PE outbreaks and in experimentally inoculated pigs, it failed to demonstrate a high diagnostic specificity since uninoculated pigs also showed positive results (Holyoake et al., 1994b). The low specificity of the test may also have resulted from the use of impure culture bacteria as antigen.

After pure *L. intracellularis* cultures became available, later ELISA developments used the pure cultures of whole *L. intracellularis* cells as antigen. A variety of whole-cell extraction methods were used for antigen preparation, such as the

Westphal hot phenol for lipopolysaccharide (LPS) extraction (Kroll et al., 2005), a sodium deoxycholate (DOC) extraction (Boesen et al., 2005), and sonication of pure culture *L. intracellularis* (So-ELISA) (Wattanaphansak et al., 2008). The sensitivities of those ELISAs were estimated to be 88.7%, 98%, and 89.8%, respectively. The specificities were estimated to be 93.7%, 98.0%, and 99.4%, respectively. Although the performances of those ELISAs were significantly better, the whole-cell base ELISA is limited in its potential to cross-react with other enteric bacteria. Moreover, it requires a live culture of bacteria for antigen preparation. Only a few laboratories around the world are capable of preparing large amounts of antigen. To avoid the difficulty of culturing live bacteria for antigen preparation, one recombinant protein FliC-ELISA has been successfully developed and validated for the detection of a *L. intracellularis*-specific antibody. However, the performance of the FliC-ELISA failed to demonstrate high sensitivity and specificity (Wattanaphansak et al., 2006). Future investigations with another recombinant protein-based ELISA should be conducted in order to develop another diagnostic test for PE diagnosis. Only one commercial whole-cell blocking ELISA system is currently available for PE diagnosis (Keller et al., 2004). Although this blocking ELISA is available for use worldwide, its performance has not been validated or compared with other serologic tests.

To date, a major limitation of all current serological tests is that none of them have the ability to differentiate between immune responses from vaccination and natural infection. Furthermore, serological results only indicate the exposure status of animals and are unable to confirm the presence or absence of disease or pathogen.

In interpreting of test results regarding the nature of the disease, it is also important to understand the limitations of the tests. Guedes (2004) suggested that if pigs are PCR-positive but serology-negative, they are either in the early stages of infection, and seroconversion has not yet developed, or the level of infection is not high enough to stimulate the seroconversion after an infection. If pigs are PCR-negative but serology-positive, it implies that either those animals no longer shed the bacteria although they have certainly been exposed to the bacteria for a while, or they continue to shed the bacteria at levels below the detection limit of the PCR assay.

## 1.6 Treatment and control

When an outbreak of PE is suspected to have occurred in the herd, the first action is to confirm that the problem is indeed caused by *L. intracellularis*, since several diseases have similar clinical signs and lesions. The diagnosis of PE infection can be confirmed using a combination of several antemortem and postmortem diagnostic tests. Pending confirmation, however, plans must be developed in order to rapidly control the progress of disease and to prevent a future outbreak. Several studies report the efficacies of antimicrobial therapy via the feed or water (McOrist et al., 1996; McOrist et al., 1997; Winkelmen et al., 1998; Walter et al., 2001), an attenuated live vaccine (Roof, 2001; Guedes and Gebhart, 2003b; Kroll et al., 2004; McOrist and Smits, 2007), passive egg antibodies (Winkelman et al., 2004), and disinfectants (Collins et al., 2000) for the treatment and control of PE in pigs.

With regard to antimicrobial treatment and control, it is crucial to select the antimicrobials most effective in inactivating the bacteria inside the animal and

eliminating the bacteria from the animal's body. This could also prevent the excretion of active bacteria which would later infect new susceptible pigs. For most bacteria, antimicrobial selection decisions are made on the basis of *in vitro* sensitivity data obtained from pure culture of the organism that caused the outbreak. However, the isolation of *L. intracellularis* from each outbreak case would be extremely difficult and very time consuming, as the standard method for isolation of *L. intracellularis* requires several weeks to months before pure culture is established (Lawson et al., 1993). Therefore, *in vitro* sensitivity results are nearly impossible to obtain in time to treat an outbreak, making it difficult for veterinarians to decide on antimicrobials to use after an outbreak occurrence.

Little information is available about *in vitro* antimicrobial sensitivities against *L. intracellularis*. This is mainly because only a few strains of *L. intracellularis* have been successfully isolated and maintained *in vitro*. It is estimated that less than 20 *L. intracellularis* isolates have been established as pure culture worldwide. Moreover, the standard antimicrobial susceptibility tests used for other organisms are not applicable for *L. intracellularis.* Only a tissue culture system, which is a complicated method both to perform and to interpret, has been used to evaluate the *in vitro* activities of some antimicrobials against a few isolates of *L. intracellularis* (McOrist et al., 1995c).

Previous *in vitro* testing of antimicrobial susceptibility showed that ampicillin, penicillin, erythromycin, difloxacin, virginiamycin, chlortetracycline, tiamulin, and tilmicosin were the most active compounds against *L. intracellularis* with minimum inhibitory concentrations (MICs) of ≤4 μg/ml. *L. intracellularis* is intermediately sensitive to lincomycin, enrofloxacin, and tylosin with MICs of 8 to 64 μg/ml. In

contrast, antimicrobials that had the lowest activities against *L. intracellularis* were aminoglycosides, apramycin, and vancomycin, with MICs > 128 µg/ml (McOrist et al., 1995c; McOrist and Gebhart, 1995). Although these studies were successful in determining the *in vitro* activity of antimicrobials against *L. intracellularis,* the results are limited because only 2 to 3 *L. intracellularis* isolates obtained from the United Kingdom were tested. Furthermore, the original tissue culture assay is time consuming and difficult to perform. Consequently, in order to expand this limited information, additional primary isolates of *L. intracellularis* from different regions and times of sample collection must be obtained and tested. Moreover, a new, reliable, and easy-to-perform *in vitro* sensitivity assay needs to be developed for use with *L. intracellularis*. This updated *in vitro* result could be used as a guideline for antimicrobial selection in the treatment and control of PE in the future. In the swine industry, the most common antimicrobials used for treatment and control of PE are chlortetracycline, tiamulin, tylosin, lincomycin, carbadox, and valnemulin. However, little is known about the *in vitro* activity of these compounds against *L. intracellularis.*

The responses of *L. intracellularis* to antimicrobials in *in vitro* conditions were similar to the results observed under *in vivo* studies. In an *in vivo* study with tetracycline, McOrist et al. (1999a) showed that no gross or histopathological lesions developed or were observed in experimental pigs fed high doses of chlortetracycline (300 or 600 ppm) 4 days prior to inoculation through 21 days post inoculation. Under field conditions, using doxytetracycline at concentrations of 125 and 250 ppm in the feed significantly reduced the number of diarrhetic pigs, fecal PCR-positive pigs, and histopathological lesions, compared to 50 ppm doxytetracycline and control pigs

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(Kyriakis et al., 2002). On the other hand, low concentrations of chlortetracycline at 100 ppm failed to reduce histopathological lesions of PE as compared to the controls (Winkelman et al., 1996b). This indicates that *L. intracellularis* responds well to high concentrations of chlortetracycline and doxytetracycline.

For *in vivo* activities of tiamulin, McOrist et al. (1996) demonstrated the "preventative" strategy by continuously feeding pigs 50 ppm tiamulin 2 days before challenge through 21 days post-challenge and demonstrated the "treatment" plan by feeding tiamulin at 150 ppm 7 days after challenge. Neither group of animals showed clinical signs and no specific lesions of PE were found in any section of intestine. Similar results were found when a low concentration of tiamulin, at 35 or 50 ppm, was used in the feed. The treated animals showed less severe macroscopic and microscopic lesions, fecal shedding, and seroconversion compared to the non-medicated control group (Schwartz et al., 1999). Tiamulin not only can be mixed with the feed, but it can also be dissolved and delivered via drinking water. Walter et al. (2001) demonstrated the use of tiamulin at 35 ppm in the feed and at 60 ppm in the drinking water. Unlike the previous two studies, feed and water medication were delivered to the infected pigs after 60% of the infected animals showed the clinical signs of PE. Pigs treated with tiamulin via water and feed had significant reductions in gross and microscopic lesions, severity of clinical signs, and prevalence of fecal shedding compared with the controls.

For *in vivo* activities of tylosin, McOrist et al. (1997) demonstrated that the use of 100 ppm tylosin in feed introduced 7 days after infection significantly inhibited gross lesions in the small intestine. In another study, tylosin was used for the treatment and control of PE after a natural outbreak. After the infected pigs were treated with 110 ppm

of tylosin in the feed for 21 days, clinical signs of infected pigs (expressed as clinical impression scores) improved more rapidly than non-medicated controls. However, the overall growth performance showed no significant difference between the two treatment groups (Veenhuizen et al., 1998). The efficacy of the tylosin for treatment and control of PE has also been evaluated using injectable and drinking water administration. Marstellar et al. (2000) demonstrated that the number of diarrhetic pigs and the severity of gross and microscopic lesions in infected pigs were significantly reduced after Tylosin 200[TM] (injection form) was applied as an intramuscular injection. Paradis et al. (2004) described the use of tylosin (Tylan[®] soluble powder) at a concentration of 83.3 ppm via drinking water. The medicated water was introduced to the infected pigs at 10 days after infection and continued for 7 days. The treatment group showed lower fecal scores, lower lesion scores, lower rates of PCR-positive tissues, and better overall growth performance compared to the untreated pigs.

For *in vivo* activities of lincomycin, McOrist et al. (2000) conducted a clinical trial to determine the efficacy of water-soluble lincomycin-spectinomycin powder against *L. intracellularis* in European pig farms. After the participating herds were confirmed positive with PE infection by PCR, the infected pigs were randomly assigned to receive the combination of lincomycin-spectinomycin (21 ppm and 42 ppm, respectively) for 7 or 14 days. Diarrhetic pigs with *L. intracellularis* infection were found to recover within 3 to 7 days after medication application. The overall growth performance of the treated groups was better than in the untreated groups. Similar results were found whether the pigs were medicated 7 or 14 days. Winkelman et al. (2002) determined the *in vivo* activity of lincomycin via feed medication against *L.*

*intracellularis* infection. Results showed that oral administration of lincomycin at 44 ppm and 110 ppm in feed for 21 days significantly reduced the number of diarrhetic pigs and improved average daily gain (ADG). However, both concentrations failed to prevent microscopic lesions, and only lincomycin at 110 ppm significantly reduced the mortality rate of infected pigs. This indicates that if lincomycin is used alone for the treatment of PE infection, a high concentration of lincomycin might be required for an adequate effect against *L. intracellularis*.

For *in vivo* activities of carbadox, Winkelman et al. (2003) conducted a gut mucosal homogenate challenge study to determine the *in vivo* efficacy of carbadox against *L. intracellularis*. Pigs were randomly assigned to receive feed with 50 ppm of carbadox 1 day before challenge through day 28 (T1), 50 ppm of carbadox on day 14 after challenge through day 28 (T2), or non-medicated feed (T3). Pigs fed 50 ppm of carbadox (T1) were completely protected from *L. intracellularis* infection. Pigs were fecal PCR- and immunohistochemistry-negative at all time points of sample collection. Fecal shedding of *L. intracellularis* was dramatically reduced in infected pigs fed 50 ppm carbadox 2 weeks after infection (T2). Overall performance and gross lesions in the treated groups were significantly different compared to the untreated group. Although carbadox demonstrated high antimicrobial activity against *L. intracellularis*, its use has been restricted in several countries due to concerns of carcinogenic toxicity.

For *in vivo* activities of valenemulin, McOrist et al. (1998) conducted a study to determine the efficacy of valnemulin for the prevention and treatment of disease. For use in prevention, valnemulin was added in the feed at 0, 25, 37.5 and 50 ppm 2 days before infection and continuously fed until day 21 post infection. For use as treatment,

valnemulin was added in the feed at 75 ppm 7 days after infection until day 21 post infection. Dose responses of gross and microscopic lesions were found in pigs treated with 25, 37.5, and 50 ppm of valnemulin. No gross lesions were found in pigs receiving 50 ppm valnemulin; however, a few microscopic lesions were observed. Moreover, no gross or microscopic lesions were found in pigs treated with 75 ppm valnemulin, and growth performance of this group was markedly improved compared with the untreated controls. Several studies have also been conducted to determine the optimum dose of valnemulin for the treatment and control of PE. Results suggest that valnemulin at 25 to 50 ppm in the feed significantly reduced clinical signs, fecal shedding, and improved growth performance of the pigs (Winkelman et al., 2000).

The timing of antimicrobial initiation in infected pigs after an outbreak is another factor that needs to be considered and investigated. Collins et al. (2001) reported that if the antimicrobials were introduced later than 14 days after infection, pigs would suffer from more severe clinical signs and lesions from which they may not easily recover. On the other hand, if the antimicrobials are applied before the initiation of the immune response, the long-term immunity needed to protect pigs from future re-infection may not develop.

The use of disinfectants is the most effective strategy for the control of *L. intracellularis* in the environment. However, no scientific evaluations of disinfectant efficacies against *L. intracellularis* under field conditions have been reported. A little information of *in vitro* validation of some disinfectants is available. In a previous *in vitro* study in which a conventional tissue culture assay was used to determine bacterial viability, *L. intracellularis* was found to be susceptible to 3.3% wt/vol quaternary

ammonium compound and 1% povidone-iodine. In contrast, the bacteria were tolerant of both 0.33% phenolic mixture and 0.0005% hydrogen peroxide/peracetic acid. Moreover, there were variations in the response to 1% potassium peroxymonosulfate and 0.001% sodium hypochlorite (Collins et al., 2000). However, this study had several limitations. The efficacies of the disinfectants were tested without the presence of water hardness or organic material. Therefore, it is possible that the bactericidal activity of the disinfectants may not have been as effective as reported *in vitro* when used in environments containing such influential factors. Additionally, the conventional tissue culture that was used in this study was time-consuming, and the assay was incapable of distinguishing between proportions of viable and non-viable bacteria after exposure to the disinfectants. Future investigation of a new method to determine the bactericidal activity of various disinfectants against *L. intracellularis* is still required. A new assay could be applied for future disinfectant evaluations to allow the selection of the most active disinfectants for the control of disease.

Increasing pig immunity is another alternative to be considered in the control of PE in swine herd. The long-term goal of using a vaccine is to prevent an outbreak from occurring in the future. To date, there is only one modified-live *L. intracellularis* vaccine (Enterisol® Ileitis, Boehringer Ingelheim Vetmedica) available for use. The effectiveness and safety of this vaccine have been validated and confirmed in a variety of experimental and field conditions (Roof, 2001; Knittel et al., 2000; Guedes and Gebhart, 2003b; McOrist and Smits, 2007). This modified-live vaccine is recommended for delivery to pigs via oral administration with clean water at least 3 weeks prior to natural *L. intracellularis* exposure. Medication in water and feed must be removed 3

days prior to and 3 days post-vaccination. Timing of the vaccination is an important factor since the vaccination requires time to generate immunity before the natural infection occurs. Therefore, it is necessary to estimate the time of infection in the production system before vaccine application. Vaccinated animals could develop long-term active immunity (humoral and cell mediated immune responses) to protect the vaccinated pigs from a virulent field strain of *L. intracellularis* (Roof, 2001). Vaccinated pigs had significantly less severe gross and microscopic lesions, less colonization and fecal excretion of the organism, and improved ADG when compared to challenge-control pigs (Kroll et al., 2004). Although the vaccine gave good protection against *L. intracellularis* infection, the mechanism of immunity development remains unclear. Further investigation into local immune response and its mechanism of action are needed.

Chicken egg antibodies have been recommended for use in swine feed to provide passive immunity against PE in pigs by preventing colonization with *L. intracellularis*. Chickens are hyperimmunized with pure culture *L. intracellularis*, and the antibodies are secreted into the eggs. The infected pigs that were fed chicken egg yolks containing anti-*L. intracellularis* antibodies had significantly increased average daily feed intake and ADG. However, there were no statistical differences found in terms of macroscopic or microscopic lesions, fecal shedding,  clinical signs, when compared among treatment groups where the dose in feed was varied (Winkelman et al., 2004).

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SECTION II: PORCINE PROLIFERATIVE ENTEROPATHY – TREATMENT AND

CONTROL

CHAPTER 2

*IN VITRO* ANTIMICROBIAL ACTIVITY AGAINST 10 NORTH AMERICAN AND

EUROPEAN *LAWSONIA INTRACELLULARIS* ISOLATES

The objective of this study was to determine the *in vitro* minimum inhibitory concentration (MIC) of antimicrobials against 10 isolates of *Lawsonia intracellularis*, the etiological agent of proliferative enteropathy (PE). Antimicrobials tested included carbadox, chlortetracycline, lincomycin, tiamulin, tylosin and valnemulin. The MIC of each antimicrobial against *L. intracellularis* was determined using a tissue culture system and was identified as the lowest concentration that inhibited 99% of *L. intracellularis* growth, as compared to the antimicrobial-free control. Each antimicrobial concentration was evaluated for both intracellular and extracellular activity against *L. intracellularis*, an obligately intracellular bacterium. When tested for intracellular activity, carbadox, tiamulin, and valnemulin were the most active antimicrobials with MICs of ≤0.5 μg/ml. Tylosin (MICs ranging from 0.25 to 32 μg/ml) and chlortetracycline (MICs ranging from 0.125 to 64 μg/ml) showed intermediate activities and lincomycin (MICs ranging from 8 to >128 μg/ml) showed the least activity. When tested for extracellular activity, valnemulin (MICs ranging from 0.125 to 4 μg/ml) was the most active against most *L. intracellularis* isolates. Chlortetracycline (MICs ranging from 16 to 64 μg/ml), tylosin (MICs ranging from 1 to >128 μg/ml), and tiamulin (MICs ranging from 1 to 32 μg/ml) showed intermediate activities. Lincomycin (MICs ranging from 32 to >128 μg/ml) showed the least activity. Our *in vitro* results showed that each *L. intracellularis* isolate had a different antimicrobial sensitivity pattern and these data can be utilized as an *in vitro* guideline for the further antimicrobial evaluation of field *L. intracellularis* isolates.

**2.1 Introduction**

Proliferative enteropathy (PE) is one of the most prevalent enteric bacterial diseases in grower and finisher pigs. The etiological agent of this disease is an obligate intracellular, gram-negative bacterium named *Lawsonia intracellularis*. The treatment of a PE outbreak on a pig farm often involves antimicrobial therapy. For many bacterial agents, appropriate antimicrobial therapy is determined through *in vitro* antimicrobial susceptibility testing results. However, since little information is available on *in vitro* antimicrobial sensitivities against *L. intracellularis* infection, the selection of an appropriate antimicrobial is difficult.

The paucity of information is due to the fact that standard antimicrobial assays are not applicable to evaluate the antimicrobial activities of most intracellular organisms since the bacterium only propagates itself inside the host cell. Therefore, most *in vitro* studies of antimicrobial activities against obligate intracellular bacteria are undertaken through a complicated cell culture system (McOrist et al., 1995c; Gnarpe et al., 1996; Ives et al., 2000; Horowitz et al., 2001). Furthermore, few strains of *L. intracellularis* have been successfully isolated and maintained *in vitro*. Of these, only 3 European isolates, 51/89, 1482/89 (NCTC 12656), and 916/91 (NCTC 12657), have been tested *in vitro* for antimicrobial susceptibilities using a tissue culture system (McOrist and Gebhart, 1995; McOrist et al., 1995c). It has been a decade since these antimicrobial activity studies of *L. intracellularis* have been reported, and no further *in vitro* studies have been published to update or expand upon the limited data existing for the antibiotic sensitivity of *L. intracellularis*. Therefore, the objective of this study was to determine the *in vitro* antimicrobial sensitivities of 10 isolates of *L. intracellularis* obtained from

both North America and Europe against 6 antimicrobial compounds that have been used for treatment and control of PE in pigs.

## 2.2 Materials and Methods

### 2.2.1 Source and preparation of antimicrobials

The following antimicrobial agents were purchased as pure chemicals: carbadox, chlortetracycline hydrochloride, lincomycin hydrochloride and tylosin tartrate (Sigma-Aldrich, Missouri, United States). Tiamulin hydrogenfumarate and valnemulin hydrochloride were supplied as pure chemicals from Novartis Animal Health (Basel, Switzerland). The stock solutions of all antimicrobial compounds were prepared to a final concentration of 2,560 μg/ml. Each antimicrobial solution was sterilized by filtration using 0.2 μm-pore size filters. Stock solution of carbadox was first dissolved with 0.1 N NaOH and then was diluted in sterile distilled de-ionized water. The stock solutions of the other compounds were dissolved directly in sterile distilled de-ionized water. All stock solutions were aliquoted into 5-ml polystyrene tubes and kept at -20$^{\circ}$C until use. Once the antimicrobials were thawed, they were used and kept refrigerated for up to 3 days. A series of 2-fold dilutions were made with the 2,560 μg/ml stock solutions, and these were then diluted 1:10 with culture medium. The resultant final concentrations of active antimicrobials were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/ml. Each concentration of antimicrobial was tested in triplicate.

**2.2.2 Bacterial strains and preparation**

A total of 10 *L. intracellularis* field strains collected between 1983 and 2006 from infected pigs in the United States and Europe were tested. Six *L. intracellularis* strains were used from the United States: PHE/MN1-00, VPB4, KKumn04, NWumn05, DBumn06 and 47216-06. Three *L. intracellularis* strains used were from the United Kingdom: LR189/5/83, 963/93 and 916/91; and one *L. intracellularis* strain used was from Denmark: D15540. All strains were stored at -72$^{o}$C until use.

All strains of *L. intracellularis* were grown in murine fibroblast-like McCoy cells (CRL 1696, American Type Culture Collection, Virginia, United States) and were maintained in a cell culture system as described previously (Guedes and Gebhart, 2003; Wattanaphansak et al, 2005). To release the bacteria, the monolayer was scraped and the cell suspension was passed 10 times through a 10-inch, 20-gauge needle. The suspension was then passed through a 5-$\mu$m filter to remove the ruptured McCoy cells. Following filtration, the purified *L. intracellularis* was centrifuged at 8000$\times$ *g* for 20 min and washed with DMEM 2 times. The bacterial pellet was finally diluted with 100 ml cell culture media composed of DMEM, 7% FBS, 1% L-glutamine, and 0.5% amphotericin B. The final concentrations of the *L. intracellularis* inoculum of tested *L. intracellularis* strains were individually estimated using a conventional serial dilution count as previously described (Guedes and Gebhart, 2003).

**2.2.3 Antimicrobial sensitivity testing**

A tissue culture system was modified from a previous study (McOrist et al., 1995c) to determine the minimum inhibitory concentration (MIC) of each antimicrobial

against *L. intracellularis*. Briefly, the frozen bacteria were thawed and grown in cell culture for at least 3 continuous passages. This was performed in order to allow the bacteria to recover from the frozen stage and allow the infection to reach 100% of the McCoy cells in the monolayer. All *L. intracellularis* isolates were tested twice and each replicate was prepared independently. Each strain of *L. intracellularis* was harvested from the monolayer of 3 infected T-175 cm$^2$ flasks as described earlier. Once the bacterial pellet was diluted with 100 ml culture medium, 100 μl of bacterial suspension was inoculated onto 1-day-old McCoy cells in 96-well tissue culture plates (Nalge Nunc International, New York, United States).

In this study, the MICs were expressed for both intracellular and extracellular activities. Intracellular MIC testing was conducted in order to measure the effect of antimicrobials on *L. intracellularis* after the bacteria had infected the enterocytes. For intracellular testing, a previously published assay was used (McOrist et al., 1995c), with minor changes to the cell line and the bacterial concentration. Briefly, one hundred μl of bacterial suspension containing approximately 10$^6$ to 10$^7$ *L. intracellularis*/ml was inoculated onto 1-day-old McCoy cells 24 hours before exposure to the antimicrobials. This permitted sufficient time for *L. intracellularis* to penetrate into the host cells prior to antimicrobial treatment. After incubation, the bacterial suspension was removed and replaced with 100 μl of fresh culture medium containing various concentrations of antimicrobials at 1, 2, and 3 days post inoculation, followed by fresh culture media on day 4 with no antimicrobial as previously described (McOrist et al., 1995c).

The extracellular MIC testing was designed with the intention of mimicking the effect of antimicrobials on *L. intracellularis* when *L. intracellularis* is free in the gut

lumen before infecting the intestinal cells. For extracellular testing, we followed a previously described approach (McOrist et al., 1995c) with minor changes to the cell line, the inoculum concentration, and the duration of incubation in the presence of the antibiotic prior to infection of the cell line. Briefly, a series of 2-fold dilutions of stock antimicrobials were added to culture medium which contained *L. intracellularis*. The suspension was incubated at $37^{o}$C in 8.0% oxygen, 8.8% carbon dioxide, and 83.2% nitrogen atmosphere for 2 hours without mixing, allowing the bacteria to be exposed directly to the antimicrobials. After incubation, one hundred μl of the bacterial suspension was transferred to infect 1-day-old McCoy cells. The medium was removed after 24 hours incubation and replaced with 100 μl of new culture medium without any antimicrobials for 3 consecutive days as previously described (McOrist et al., 1995c). The last row and last column of each infected plate were maintained as the control cultures so that each antimicrobial concentration had a duplicate well containing media with no added antimicrobial. Following the media removal each day, the infected plates were exposed to hydrogen gas and the plates were then kept at $37^{o}$C for 5 days in an incubator with 8.0% oxygen, 8.8% carbon dioxide and 83.2% nitrogen as the atmosphere.

After 5 days incubation, supernatant from the infected plates was removed and the cell culture monolayer was fixed with 100 μl of cold 50% acetone and 50% methanol for 1 min. 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 (Guedes et al., 2002b). Briefly, the fixed plates were re-hydrated with distilled water at $37^{o}$C for 60 min. The water was

discarded and 50 μl of rabbit polyclonal antibody (titer >1:40,000), which was diluted in skim milk buffer (5% (wt/vol) skim milk in phosphate buffer saline (PBS) with 0.05% Tween 20) to 1:5,000, was used as a primary antibody. After 30 min incubation at 37°C, the plates were then washed 4 times with PBS. Fifty μl of anti-rabbit IgG horseradish peroxidase conjugate (SouthernBiotech, Alabama, United States) diluted 1:2,000 in skim milk buffer was added to each well. The plates were then incubated again at 37°C for 45 min and washed 4 times with PBS. One hundred μl of freshly prepared chromogen solution (500 μl of 3-amino-9-ethyl-carbazol, 9.5 ml of acetate buffer, 5 μl of 30% hydrogen peroxide) was applied and incubated at room temperature for 20 min. The stained plates were finally washed with distilled water 4 times before being allowed to air-dry.

The *L. intracellularis* proliferation was evaluated by counting the number of heavily infected cells (HIC) (McOrist et al., 1995c) in each well using an inverted microscope (Olympus, Tokyo, Japan) with a 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. A comparison was then made in which the number of HICs in each well was expressed as a percentage compared to the average HICs of the control wells. The intracellular and extracellular MIC endpoints for each antimicrobial in this study were defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation in the McCoy cells after 5 days of incubation as defined previously (McOrist et al., 1995c). These inhibitions were indicated by the percentage of HIC of each antimicrobial concentration compared to the antimicrobial-free control.

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**2.2.4 Data analysis**

The MIC endpoints of each antimicrobial were determined using the median value from a set of triplicate wells. MIC assays were performed in duplicate from independent bacterial preparations, and the duplicate MIC endpoints were expressed for each antimicrobial for each isolate. When the percentage of HIC of *L. intracellularis* in the antimicrobial-free control was less than 50%, the MIC tests for that *L. intracellularis* strain were repeated.

**2.3 Results**

The intracellular and extracellular MIC values for antimicrobials used against the 10 *L. intracellularis* isolates in the present study are shown in Table 2.1. The HIC of *L. intracellularis* in the McCoy cell and percentage of infection compared to the control are demonstrated in Figure 2.1.

The concentrations of *L. intracellularis* inocula were between $1.2x10^6$ and $3.4x10^7$ *L. intracellularis* organisms/ml, and each isolate had a range of less than one log between the two replicates. For *L. intracellularis* isolates from the U.S. (n=6), the intracellular activity results showed that carbadox, tiamulin, and valnemulin displayed the highest activity against all *L. intracellularis* isolates with MICs of $\leq 0.5$ µg/ml. Chlortetracycline and tylosin showed moderate activity against *L. intracellularis* with MIC ranges of 0.125 to 64 µg/ml and 0.25 to 32 µg/ml, respectively. Lincomycin showed the lowest activity against most *L. intracellularis* isolates with an MIC range from 16 to >128 µg/ml. The extracellular activity results showed that only valnemulin had high activity against *L. intracellularis* with MICs ranging from 0.125 to 4 µg/ml.

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Antibiotics with moderate activities against *L. intracellularis* included carbadox with an MIC range of 4 to 32 μg/ml, chlortetracycline with an MIC range of 32 to 64 μg/ml, tiamulin with an MIC range of 1 to 32 μg/ml, and tylosin with an MIC range of 1 to >128 μg/ml. All *L. intracellularis* isolates from the U.S. had the lowest extracellular activity to lincomycin with MICs of >128 μg/ml.

MIC results for the European isolates (n=4) were similar to the U.S. isolates in that, for the intracellular MICs, carbadox, tiamulin, and valnemulin had the highest activity against *L. intracellularis* with MICs of 0.125 μg/ml. Antimicrobials with moderate activities included chlortetracycline with an MIC range of 0.25 to 16 μg/ml, lincomycin with an MIC range of 8 to 64 μg/ml, and tylosin with an MIC range of 0.5 to 2 μg/ml. The extracellular activity results showed that valnemulin had the highest activity against *L. intracellularis*; all isolates had MICs of ≤ 0.25 μg/ml. The antimicrobials that had moderate activity were carbadox with an MIC range of 1 to 4 μg/ml, chlortetracycline with an MIC range of 16 to 64 μg/ml, tiamulin with an MIC range of 1 to 4 μg/ml, and tylosin with an MIC range of 2 to 16 μg/ml. The antimicrobial that showed the lowest activity against *L. intracellularis* was lincomycin with MICs of 32 to >128 μg/ml.

## 2.4 Discussion

The standard liquid MIC and agar plate diffusion assays that are regularly used for determining antimicrobial activity against swine enteric bacteria are not appropriate for obligate intracellular bacteria since these bacteria proliferate themselves only when

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inside the host cells. Although methodologies for determining antimicrobial sensitivity of intracellular organisms have been developed, the methods and interpretations of their results have not been standardized to be uniformly accepted. In this study, the practicality of assessing the *in vitro* antimicrobial activity against *L. intracellularis* was demonstrated using the tissue culture system, which was modified from a previous study (McOrist et al., 1995c).

Previous studies that tested various antimicrobials against *L. intracellularis* strains used only 3 strains from the United Kingdom, mainly due to the limitation of the strains available and the difficulty of the laboratory techniques for maintaining and culturing *L. intracellularis* (McOrist and Gebhart, 1995; McOrist et al., 1995c). In this study 6 different antimicrobials against 10 *L. intracellularis* strains were evaluated. The previously published MIC methods were also modified in several ways. The major modifications to this previous study were the use of McCoy cells versus IEC-18 cells and the increase of the *L. intracellularis* inoculum concentration. The McCoy cells were used in this study because *L. intracellularis* grows better in these cells, allowing the achievement of 100% HIC in the unmedicated control wells and, therefore, increasing the accuracy of the MIC calculations. Furthermore, McCoy cells were more recently recommended for use in determining the antimicrobial activity for intracellular organisms (Suchland et al., 2003). The concentration of the *L. intracellularis* inoculum was increased in this study because preliminary results showed that the inoculum concentration at $10^4$ organisms/well yielded an infection rate of less than 50% (data not shown). Ninety-six-well tissue culture plates were also used, thus allowing the testing of a larger number of antimicrobial agents over various concentrations at the same time.

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In this study, the extracellular and intracellular MICs for *L. intracellularis* were determined in an effort to mimic *L. intracellularis* infections in which the bacteria would be exposed to antimicrobials before and after invasion into intestinal cells. The results of the intracellular and extracellular MICs obtained from 2 different batches of each *L. intracellularis* strain demonstrated that the assay was reproducible, as the median MIC from the 2 replicates was always within a 2-fold dilution, determined by assessing whether any duplicate was more than 2-fold dilution away from the log2 mean MIC (Table 2.1). To determine the extracellular activity, the bacteria were exposed to the antimicrobial agents for 2 hours before being transferred to the enterocytes. This was done because *L. intracellularis* can rapidly penetrate host cells, with estimates ranging from 10 to 180 min (Lawson et al., 1993; McOrist et al., 1995b). Without this 2-hour incubation, it would have been possible for the *L. intracellularis* to invade the host cell before being affected by the antimicrobial. These extracellular MIC results showed that, of the antimicrobials tested here, only valnemulin was highly active.

One European isolate (916/91 or NCTC 12657) that was tested in the previous study (McOrist et al., 1995c) was also retested for antimicrobial activity in this study. Unfortunately, individual MIC endpoints were not shown for individual isolates in previous data. Therefore, a direct comparison of the MIC endpoint of the current study to the earlier study is difficult to perform. With the exception of tylosin and tiamulin (intracellular MIC), the MIC results of the tested antimicrobials from the earlier study (McOrist et al., 1995c) were always within the range of a 2-fold dilution compared to our results.

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Like other intracellular bacteria, once the *L. intracellularis* organisms have internalized themselves inside the host cells, they are shielded by the host cell membrane. For this reason, the antimicrobial compounds cannot be considered to be in direct contact with their bacterial targets. The intracellular activities of the antimicrobials are of critical concern as many parameters govern their activity, including (i) the balance between influx and efflux, (ii) the pharmacokinetic and pharmacodynamic properties of the antimicrobial, (iii) the state of bacterial responsiveness, (iv) the physicochemical condition at the infection site, and (v) the degree of cooperation with host defenses (Carryn et al., 2003). Thus, our study also determined the intracellular activity of antimicrobials against *L. intracellularis*.

The intracellular activity results showed that carbadox, tiamulin, and valnemulin were the most active compounds against all 10 *L. intracellularis* isolates originating from the U.S. and Europe, inhibiting intracellular activity at <0.5 μg/ml. However, intracellular activities of some *L. intracellularis* isolates from the U.S. and Europe were also inhibited by chlortetracycline and tylosin, while lincomycin showed the lowest *in vitro* activity against most *L. intracellularis* isolates. The intracellular MIC results from previous reports found that ampicillin, chlortetracycline, difloxacin, erythromycin, penicillin, and virginiamycin were the most active compounds against the 3 *L. intracellularis* isolates tested. In contrast, bacitracin-zinc, avoparcin, and all aminoglycoside and aminocyclitol groups showed low activity against *L. intracellularis* in both intracellular and extracellular activity tests (McOrist et al., 1995c). Of these antimicrobials, this study only tested the activity of chlortetracycline, as the others are not commonly used for PE treatment and control in pigs.

Currently there are no antimicrobial MIC breakpoints for intracellular organisms using a tissue culture system; therefore, interpretations of the sensitivity data are complicated. These data may only be used as a guide to determine which antimicrobials would be most effective in treating *L. intracellularis* infections *in vivo* for various reasons. First, it is unknown how the *in vitro* assay compares to *in vivo L. intracellularis* infections. Second, it is unknown what concentration of each antimicrobial can be attained at the site of *L. intracellularis* infection. This concentration that can be achieved at the site of infection is critical in determining whether a specific isolate is susceptible or resistant. Finally, it is unknown whether the antimicrobial would have the greatest effect on *L. intracellularis* while the bacteria are extracellular or intracellular. The data can be used, however, to predict the utility of the antibiotic. If there is no diversity in activity levels and yet the MIC is very low, such as the observed variation in carbadox intracellular MIC values, then this antibiotic might function very well. If there is a large range of MICs, such as was observed with chlortetracycline intracellular MIC levels, then this could imply that some *L. intracellularis* isolates will have less sensitivity to that antibiotic.

From our results, extracellular MICs for all antimicrobials were higher than the intracellular MICs, while the previous report showed that both of them were similar (McOrist et al., 1995c). One explanation for this difference may be the effect of contact time with the antimicrobials. The extracellular activity assay was designed to have less contact time than the intracellular activity assay; i.e., 1 day compared to 3 consecutive days. This unequal contact time was due to the fact that *L. intracellularis* can penetrate into the cells within 24 hours (Lawson et al., 1993; McOrist et al., 1995b). By

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definition, the extracellular MIC is the effect of the antimicrobial on *L. intracellularis* before the bacteria enter the cells and, therefore, only 1 day of exposure time to the antimicrobial can be used to determine the extracellular MIC. A fraction of the *L. intracellularis* could have survived the single extracellular treatment and subsequently returned to active phase upon the disappearance of the antimicrobials. This could suggest that a single dose antimicrobial treatment may be insufficient to inhibit the growth of *L. intracellularis*. Another potential explanation for the higher extracellular MIC is the potential accumulation of antimicrobial inside the cells, making the intracellular concentration much higher than the extracellular concentration. For instance, macrolides such as erythromycin and azithromycin can accumulate intracellularly 4- to 10-fold and 40- to 300-fold higher than extracellular levels, respectively (Carryn et al., 2003). The increased intracellular concentrations could enhance the chemotherapeutic activity of antimicrobials against intracellular bacteria. In contrast with the macrolides, ß-lactams, aminoglycosides and lincomycin are poorly accumulated intracellularly (Carryn et al., 2002; Carryn et al., 2003). These accumulations are dependent upon the cellular pharmacokinetic activity of each drug. Although this study did not determine the antimicrobial concentration inside the cells, the intracellular MIC results seemed to show that most of the antimicrobials tested can penetrate and bind to internalized *L. intracellularis* to exert their chemotherapeutic action.

Although several different cell lines have been used to grow and maintain *L. intracellularis*, including McCoy cells (Wattanaphansak et al., 2005), rat small intestinal cells (IEC-18), piglet intestinal epithelial cells (IPEC-J2) (McOrist et al.,

1995b), and human epithelial cells (HEp-2) (Koyama et al., 2006), the direct effect of cell line on the MIC variations for *L. intracellularis* have not been determined. McCoy cells were used in this study because *L. intracellularis* grows very well in these cells, allowing a 100% infection rate of non-antimicrobial treated controls. Suchland et al. (2003) reported that some antimicrobials had significant variation on the MIC endpoint when they were tested against *Chlamydia* species using different cell lines. Based on the most reliable and consistent results, McCoy and HEp-2 cell lines were recommended for use in determining antimicrobial susceptibility for *Chlamydia* species.

Interestingly, one observation from this study was that the U.S. *L. intracellularis* isolates tended to have higher intracellular and extracellular MICs than the European *L. intracellularis* isolates. Additional *L. intracellularis* isolates from the U.S. and Europe will need to be tested before drawing any conclusions about geographic differences and antimicrobial sensitivities changing over time. This current study evaluated most of the *L. intracellularis* isolates currently available globally and obtained isolates from 2 different continents, including new clinical isolates (low passage) and well-established (high passage) *L. intracellularis* isolates. This was important because intracellular bacteria maintained under a tissue culture system for a long time have the potential to develop genomic mutations that could potentially impact their antimicrobial sensitivities (Drancourt and Raoult, 1993). Therefore, these results represent the most comprehensive *L. intracellularis* MIC study to date. Further *in vivo* studies should be conducted to confirm the antimicrobial efficacies.

Our *in vitro* data greatly expand the antimicrobial MIC information for *L. intracellularis*. Based on our *in vitro* results, it is clear that *L. intracellularis* isolates

have a diversity of antimicrobial sensitivity patterns. Because it is unlikely that *L. intracellularis* will be isolated and tested during a PE outbreak, our data can serve as an *in vitro* guideline for the range of antimicrobial responses of *L. intracellularis*. Based on this guideline, we would predict carbadox, tiamulin and valnemulin to be the most active antimicrobials, chlortetracycline and tylosin to be intermediately active, and lincomycin to be the least active antimicrobial against *L. intracellularis*.

## 2.5 Acknowledgements

**Figure 2.1** An example of an MIC endpoint of tiamulin against *L. intracellularis* strain NWumn05. Photographs of McCoy cells infected with *L. intracellularis* strain NWumn05 growing in the presence of tiamulin with concentrations ranging from 0.5 μg/ml to 128 μg/ml. After 5 days of incubation, the infected cells were fixed with cold acetone:methanol (1:1) and the infected plates were stained with IPMA protocol using rabbit polyclonal antibody. The number of heavily infected cells (HICs) in each concentration was counted and compared to antimicrobial-free controls. There was no LI 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 LI strain is 8 μg/ml.
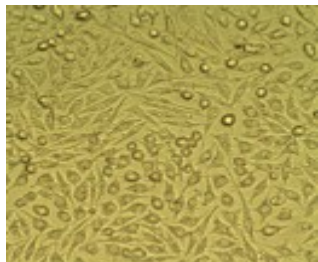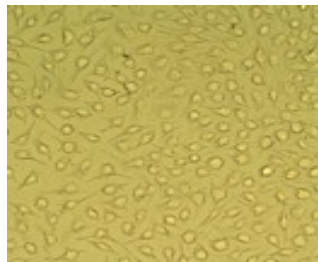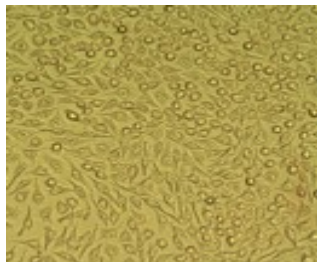
| 128 µg/ml | 64 µg/ml | 32 µg/ml | 16 µg/ml | **8 µg/ml** |
| 4 µg/ml | 2 µg/ml | 1 µg/ml | 0.5 µg/ml | Control |
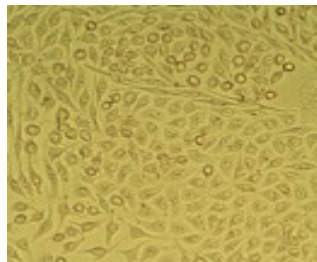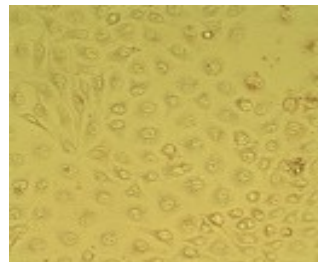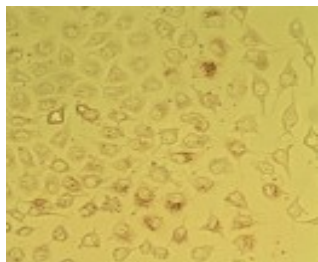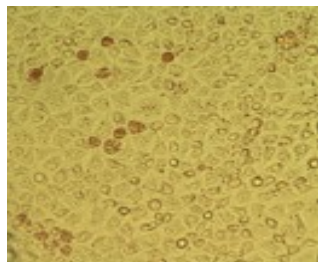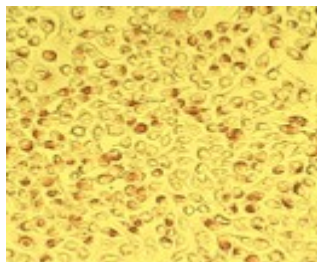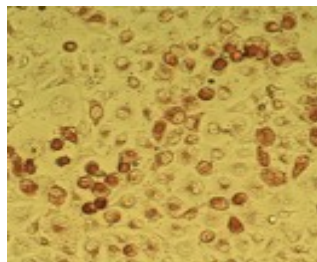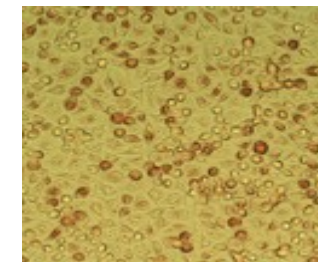
**Table 2.1** Summary of intracellular and extracellular MIC endpoints for 6 antimicrobial agents against 10 *L. intracellularis* isolates, six obtained from North America and 4 from Europe, measured by using tissue culture system with 5 days of incubation. Each strain of *L. intracellularis* was tested twice and the bacteria were prepared independently for each replicate.

| Strains of LI | Country of origin | Year | No. of passage | Antimicrobial agents (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Carbadox | | Chlortetracycline | | Tylosin | | Lincomycin | | Tiamulin | | Valnemulin | |
| | | | | Intra MIC[a] | Extra MIC[b] | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC |
| PHE/MN1-00 | USA | 2000 | 169 | 0.125 | 16 | 8 | 64 | 8 | 64 | >128 | >128 | 0.125 | 4 | 0.125 | 0.25 |
| | | | 170 | 0.25 | 16 | 4 | 32 | 2 | 64 | >128 | >128 | 0.125 | 8 | 0.125 | 0.25 |
| VPB4 | USA | 1991 | 165 | 0.25 | 32 | 4 | 64 | 8 | 128 | >128 | >128 | 0.125 | 32 | 0.125 | 2 |
| | | | 166 | 0.25 | 32 | 16 | 64 | 32 | 128 | >128 | >128 | 0.5 | 8 | 0.125 | 0.25 |
| KKumn04 | USA | 2004 | 17 | 0.125 | 4 | 32 | 32 | 0.5 | 1 | 16 | >128 | 0.125 | 1 | 0.125 | 0.125 |
| | | | 18 | 0.125 | 4 | 16 | 64 | 0.25 | 1 | 16 | >128 | 0.125 | 1 | 0.125 | 0.125 |
| NWumn05 | USA | 2005 | 21 | 0.125 | 16 | 64 | 64 | 8 | >128 | >128 | >128 | 0.125 | 16 | 0.125 | 4 |
| | | | 22 | 0.125 | 8 | 64 | 64 | 4 | 128 | >128 | >128 | 0.125 | 8 | 0.125 | 1 |
| DBumn06 | USA | 2006 | 8 | 0.125 | 4 | 0.125 | 32 | 4 | 128 | >128 | >128 | 0.125 | 4 | 0.125 | 0.25 |
| | | | 9 | 0.125 | 4 | 0.125 | 32 | 4 | 128 | >128 | >128 | 0.125 | 8 | 0.125 | 0.25 |

**Table 2.1** Continued.

| Strains of LI | Country of origin | Year | No. of passage | Antimicrobial agents (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Carbadox | | Chlortetracycline | | Tylosin | | Lincomycin | | Tiamulin | | Valnemulin | |
| | | | | Intra MIC[a] | Extra MIC[b] | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC | Intra MIC | Extra MIC |
| 47216-06 | USA | 2006 | 7 | 0.125 | 8 | 64 | 64 | 2 | 64 | >128 | >128 | 0.125 | 8 | 0.125 | 0.5 |
| | | | 8 | 0.125 | 8 | 64 | 64 | 2 | 64 | >128 | >128 | 0.125 | 4 | 0.125 | 0.5 |
| D15540 | Den | 1998 | 19 | 0.125 | 4 | 0.25 | 64 | 1 | 4 | 32 | >128 | 0.125 | 4 | 0.125 | 0.125 |
| | | | 20 | 0.125 | 4 | 0.25 | 32 | 0.5 | 2 | 16 | 128 | 0.125 | 2 | 0.125 | 0.125 |
| LR 189/5/83 | UK | 1983 | 14 | 0.125 | 1 | 0.5 | 64 | 1 | 16 | 16 | >128 | 0.125 | 4 | 0.125 | 0.125 |
| | | | 15 | 0.125 | 1 | 0.5 | 64 | 1 | 16 | 16 | >128 | 0.125 | 4 | 0.125 | 0.125 |
| 963/93 | UK | 1993 | 35 | 0.125 | 1 | 16 | 32 | 1 | 4 | 8 | 64 | 0.125 | 2 | 0.125 | 0.25 |
| | | | 36 | 0.125 | 1 | 8 | 32 | 1 | 4 | 8 | 64 | 0.125 | 2 | 0.125 | 0.25 |
| 916/91 | UK | 1991 | 16 | 0.125 | 1 | 8 | 64 | 2 | 4 | 64 | 64 | 0.125 | 2 | 0.125 | 0.125 |
| | | | 17 | 0.125 | 1 | 2 | 16 | 0.5 | 2 | 8 | 32 | 0.125 | 1 | 0.125 | 0.125 |

USA= the United States of America; Den = Denmark; UK= United Kingdom [a]The intracellular MIC and [b]extracellular MIC was defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation, compared to antimicrobial-free control.

CHAPTER 3

*IN VITRO* ASSESSMENT OF THE EFFECTIVENESS OF POWDER

DISINFECTANT (STALOSAN® F) AGAINST *LAWSONIA INTRACELLULARIS*

USING TWO DIFFERENT ASSAYS

The objective of this study was to determine the *in vitro* efficacy of Stalosan[®] F, a mixed chemicals and heavy metal disinfectant, against 2 strains of *Lawsonia intracellularis* using both a modified tissue culture and a direct count method. For testing as a powder, 1g, 0.5g, or 0.25g of Stalosan[®] F was applied to bacterial solutions spread into sterile dishes. For use as an aqueous suspension, Stalosan[®] F was prepared to final concentrations of 1%, 4%, 8%, 16%, and 32%. In both applications, *L. intracellularis* were exposed to Stalosan[®] F for 0.5, 1, 2, and 4 hours. Results showed that both strains were similar in their susceptibilities to Stalosan[®] F. The modified tissue culture assay showed no detectable *L. intracellularis* in cell culture after exposure to all levels of Stalosan[®] F powder for 0.5h. Furthermore, the number of viable bacteria was markedly reduced in the aqueous concentration of 4% and no *L. intracellularis* was detected at concentrations of $\geq$ 8% for 0.5h. Using the direct count method, the detection of live bacteria was less than 1% after exposure to the powder for 0.5h. After exposure to the aqueous form, the number of viable bacteria killed was over 99% in concentrations of $\geq$ 16% compared to the controls. Our results indicate that Stalosan[®] F in both powder and suspension forms is able to inactivate over 99% of *L. intracellularis* after 30 min of exposure. Furthermore, both laboratory methods can be used to determine the effect of disinfectants on *L. intracellularis* viability.

**3.1 Introduction**

*Lawsonia intracellularis*, a gram-negative obligately intracellular bacterium, is the etiological agent of proliferative enteropathy (PE) in pigs. This disease mainly affects growing and finishing pigs resulting in a variety of clinical signs such as chronic diarrhea, slow growth, bloody diarrhea, and sudden death (Lawson and Gebhart, 2000).

To date, there are limited data on the effectiveness of disinfectants against *L. intracellularis*. This is mainly due to the difficulty in finding good methods to measure the efficacy of disinfectants against an obligately intracellular bacterium. Only one study has been conducted and used a conventional tissue culture method to measure the viability status of *L. intracellularis* after exposure to some disinfectants (Collins et al., 2000). The limitations of that method included time-consuming procedures and inability to distinguish between proportions of viable or non-viable bacteria. Recently, a modified tissue culture method, an assay that cultures bacteria in 96-well tissue culture plates, has been developed for use in serological diagnosis (Guedes et al., 2002b) and for the determination of the *in vitro* antimicrobial activity (Chapter 2) of *L. intracellularis*. This modified tissue culture method allows the testing of a large number of compounds over various concentrations at the same time. Currently, the viability of *L. intracellularis* in a mixed bacterial population can be directly measured using a direct count method with specific fluorescence staining (Wattanaphansak et al., 2005). Both assays make it possible to evaluate the effectiveness of disinfectants against *L. intracellularis* using bacterial viability as an indicator.

Stalosan® F (Stormollen, Tureby, Storstrom, Denmark), a powder disinfectant mainly composed of phosphate compounds (85%), copper sulfate (2.5%), ferrous

sulfate (2.1%), active chlorine (0.25%), perica oil (0.05%), and al-silicate (10.1%), is indicated for use in livestock farms for reducing the number of microorganisms in the environment, absorption of moisture, and reduction of ammonia production. According to the manufacturer, the compound is recommended to be applied directly on the floor at a concentration of 50g per square meter for reducing the number of microorganisms in the environment. Although the mode of action of Stalosan® F is not fully understood, it has been shown that the numbers of pathogenic bacteria and viruses were significantly reduced when those organisms were exposed to Stalosan® F (Methling et al., 1997).

The susceptibility of *L. intracellularis* to Stalosan® F has not been reported. Therefore, the objective of this study was to evaluate the bacteriocidal activity of Stalosan® F against *L. intracellularis* using both the modified tissue culture method and the direct count method.

## 3.2 Materials and Methods

### 3.2.1 Micro-organism strains and preparations

*L. intracellularis* strains VPB4 and PHE/MN-01, which were isolated from proliferative hemorrhagic enteropathy infected pigs in the United States in 1991 and 2000, respectively (Guedes and Gebhart, 2003a), were used throughout this study. Each isolate was prepared independently and tested twice. Both isolates were grown, maintained, and harvested as described previously (Guedes and Gebhart, 2003a; Wattanaphansak et al., 2005). Briefly, stock bacteria were harvested from 10 infected T-

175 flasks after 7 days of growth in Murine fibroblast-like McCoy cells. The supernatant of each flask was discarded and only organisms in the McCoy cells were harvested. The intracellular bacteria were freed by exposing the monolayer of each flask to 15 ml of 0.1% KCl solution for 10 min at $37^{\circ}$C. Then the infected cells were harvested using a cell scraper, and the cell suspension was passed through a 10-inch, 20-gauge needle 20 times. The bacterial solution was filtrated through 5.0 μm filters to separate the bacteria from the debris of ruptured McCoy cells. The bacterial suspension was centrifuged at 8,000 g for 20 min and the pellet was re-suspended with 15 ml Dulbecco's Modified Eagle Medium (DMEM). The final concentration of *L. intracellularis* was quantified using a direct count staining procedure as described previously (Guedes and Gebhart, 2003a; Chapter 2).

### 3.2.2 Disinfectants and test procedures

Two forms of Stalosan® F preparations, a powder disinfectant and an aqueous suspension, were used for testing. For use as a powder, Stalosan® F was tested at 3 final concentrations, which were 2x, 1x, and 0.5x of recommended dosages (x= dose recommended on label). Three hundred μl of bacterial solution containing approximately $10^8$ *L. intracellularis*/ml were added in duplicate and spread gently on 10x10 cm square sterile Petri dishes. Then, 1g, 0.5g, or 0.25g of Stalosan® F powder was applied evenly to cover the entire surface of the Petri dish. These yielded final concentrations of Stalosan® F equivalent to 100 g/m$^2$, 50 g/m$^2$, and 25 g/m$^2$, respectively.

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For testing as an aqueous suspension, Stalosan® F was prepared to final concentrations of 1%, 4%, 8%, 16%, and 32% in Dulbecco's Modified Eagle Medium (DMEM). The suspension was mixed thoroughly and 8 ml of each concentration was aliquoted in duplicate into 15 ml tubes. Then 300 µl of $10^8$ *L. intracellularis*/ml was added to each aliquot.

In both applications, *L. intracellularis* was exposed to Stalosan® F for 0.5, 1, 2, and 4 hours at room temperature. The controls for each time point were live *L. intracellularis* in DMEM without exposure to Stalosan® F and dead *L. intracellularis* in which the bacteria were exposed to isopropyl alcohol for 30 min (Wattanaphansak et al., 2005). After incubation, the powder in the Petri dishes was washed with 8 ml DMEM and the suspension was immediately transferred to 15 ml tubes. The bacteria in both applications were separated from the powder by passing the suspension through 5 µm filters into 2 ml microcentrifuge tubes and centrifuged at 10,000 rpm for 3 min. The pellet was washed twice with sterile distilled water. After the final wash, half of the pellet was re-suspended with 2 ml of sterile distilled water for enumeration by the direct count method by staining the bacteria with Live/Dead® BacLight™ (Wattanaphansak et al., 2005). The other half was re-suspended with 2 ml of *L. intracellularis* culture media (DMEM + 7% FBS + 0.5% Amphotericin B + 0.5% L-glutamine + 25 µg/ml Neomycin) and the bacterial suspension was transferred to infect 1-day-old McCoy cells in the modified tissue culture method.

### 3.2.3 Bacterial survival assay

The percentage of *L. intracellularis* surviving after exposure to the disinfectant was assessed using the direct count method and the modified tissue culture method. The direct count method was conducted using Live/Dead® BacLight™ stain as described in a previous study (Wattanaphansak et al., 2005). Briefly, Live/Dead® BacLight™ stain was used and prepared per manufacturer recommendation. Equal volumes of SYTO-9 and propidium iodine (PI) were freshly mixed and used as a working solution. One µl of mixed solution was added to 2 ml of each tested sample solution. After 15 min incubation in the dark at room temperature, the stained samples were filtrated through 0.2-µm Nuclepore black polycarbonate filters. Then, each filter was placed on a glass slide with 1 drop of BacLight® mounting oil and covered with a clear glass coverslip. In this study, green fluorescent cells of live bacteria stained with SYTO-9 were counted. The number of live bacteria for each sample was estimated from a count of 5 randomly chosen microscopic fields (>400 cells). All sample counting was performed using an inverted epifluorescence microscope with a 100-W mercury light source. Images were captured with an Insight digital camera and the number of live bacteria was counted with Image Pro Plus V.6 software.

The modified tissue culture method was performed as described previously (Chapter 2) to determine *L. intracellularis* viability. Briefly, 100 µl of the final bacterial suspension was transferred to 1-day-old McCoy cells in 96-well tissue culture plates (Nalge Nunc International, New York, USA), eight wells per sample per time point. After 24 hours of incubation, the culture medium was removed and replaced with new culture medium every 2 days. After 5 days of incubation, the infected plates were

harvested and stained using a modified immunoperoxidase monolayer procedure with rabbit polyclonal antibody as described previously (Guedes et al., 2002b; Chapter 2).

The effectiveness of Stalosan® F was determined by evaluating the number of heavily infected cells (HICs), which were defined as the relative number of cells that were infected with the surviving *L. intracellularis* after exposure to the Stalosan® F. These numbers were used as a live *L. intracellularis* indicator. Cells were considered to be HICs when the number of intracellular bacteria was over 30 *L. intracellularis* per cell (McOrist et al., 1995c). To assess the number of HICs, the stained plates were counted under an inverted light microscope with a 20X objective lens.

### 3.2.4 Scanning electron microscopy (SEM)

For SEM observations, *L. intracellularis* was exposed to 0.5 g of Stalosan® F powder and 16% of Stalosan® F suspension for 30 min. The bacteria were then filtered through a 5 micron filter and washed with PBS twice. The samples were fixed with 2.5% glutaraldehyde in phosphate buffer saline (PBS) for 1 hour at room temperature. After 3 washes with PBS, the bacterial cells were fixed with 1% osmium tetroxide in PBS and washed 3 more times with PBS. The bacterial cells were dehydrated with increasing concentrations of ethanol (25%, 50%, 75%, 95%, and 100%) and dried in a Balzer Critical Point Dryer 010® unit. The fixed bacteria were coated with a thin film of gold-palladium and examined using a VPSEM-Hitachi S-3500N scanning electron microscope.

**3.2.5 Data analysis**

The number of HIC and the number of green fluorescent bacteria in each treatment of Stalosan® F were expressed as percentages as compared to controls. The correlation between the modified tissue culture method and the direct count method was estimated with Spearman's coefficient of rank correlation using the MedCalc® version 9.1.0.1 software.

**3.3 Results**

The results (Figures 3.1 and 3.2) showed that both strains of *L. intracellularis* were similar in their susceptibilities to both powder and aqueous suspension of Stalosan® F. The modified tissue culture method was used to determine the surviving population and most of the McCoy cells in live control wells were heavily infected with *L. intracellularis,* indicating that the percentage of *L. intracellularis* infection was close to 100%. In contrast, no HICs were detected in the cell culture after exposure to 0.25, 0.5, and 1 g/cm$^2$ of Stalosan® F for 30 min, indicating 100% inactivation compared to the live control (Figure 3.1A and Figure 3.3B).

When Stalosan® F was tested as an aqueous suspension, the number of HICs decreased with increased concentrations of Stalosan® F and increased exposure time. The viability of *L. intracellularis* decreased to approximately 65% after exposure to 1% of Stalosan® F concentration for 30 min. The surviving population of *L. intracellularis* was markedly reduced in the aqueous concentration of 4% and no *L. intracellularis* was detected in the cell culture at a concentration of ≥ 8% for 30 min (Figure 3.1B). Under

these conditions, both the powder and suspension forms of Stalosan® F have a bactericidal effect on *L. intracellularis*.

Using the direct count method to determine *L. intracellularis* viability, approximately 95% of live control bacteria exhibited a green fluorescence of SYTO-9. This indicated live bacteria with an intact cell membrane. In contrast, a small percentage of mixed bacterial populations illustrated a red fluorescence of PI, indicating dead bacteria with a damaged cell membrane (Figure 3.3A). After 30 min of exposure to Stalosan® F powder, the number of live bacteria progressively decreased to less than 1% in all tested concentrations of powder Stalosan® F (Figure 3.2A). In contrast, more red fluorescent bacteria were observed. Interestingly, the concentrations of both bacterial populations sharply decreased after exposure to all tested concentrations of Stalosan® F, as compared to the control. These results indicated that the bacterial structure might be damaged or lysed after contact with Stalosan® F.

Similar results were found when *L. intracellularis* was exposed to an aqueous suspension of Stalosan® F. The viable populations of *L. intracellularis* decreased with increased concentrations of Stalosan® F in the suspension. The number of live bacteria was approximately 30% after exposure to 1% of Stalosan® F for 30 min and less than 1% in concentrations of $\geq$ 16%, with more than 99% of *L. intracellularis* killed when compared to the controls (Figure 3.2B).

In this study, the results obtained from the modified tissue culture method were similar to those from the direct count method. There was a significantly positive correlation between both assays ($r^2 = 0.76$, p=0.001), indicating a good agreement between the modified tissue culture method and the direct count method.

The morphological appearance of *L. intracellularis* after 30 min of exposure to Stalosan® F is shown in Figure 3.4. Electron microscopy showed the presence of flagellar components on untreated *L. intracellularis* cells (Figure 3.4A) and the cell wall of the bacterium seemed cloudy and intact. After exposure to Stalosan® F aqueous suspension at concentrations of 4% (Figure 3.4B), 16% (Figure 3.4C), and 0.5g powder (Figure 3.4D) for 30 min, the bacterial cell wall became more translucent, indicating damage to the cell wall.

## 3.4 Discussion

The use of chemical disinfectants in swine facilities is considered a first line of defense against virus, bacteria, and parasite infection. There are many commercial disinfectants available on the market, and it is necessary to evaluate the bactericidal activities of disinfectants against *L. intracellularis* to select an efficacious one for the control of *L. intracellularis* infection. However, the effect of disinfectants on *L. intracellularis* is very difficult to measure *in vitro*. Unlike other bacteria, *L. intracellularis* is an organism that propagates itself only inside the enterocyte. Cell-free culture methods have not been successfully established and so there are no standard *in vitro* assays for assessing the efficacy of disinfectants against *L. intracellularis*. In this study, we compared 2 systems, the modified tissue culture method and the direct count method, for evaluating the efficacy of Stalosan® F in killing *L. intracellularis*.

The viability status of bacteria is used as an indicator for determining a disinfectant's efficacy (Lisle et al., 1999; Collins et al., 2000; El-Naggar et al., 2001; Jolivet-Gougeon et al., 2006). Previously, the viability status of *L. intracellularis* was

measured using live animal models (Collins et al., 2000; Guedes and Gebhart, 2003a), cell culture systems such as conventional tissue culture (McOrist et al., 1995c; Collins et al., 2000), modified tissue culture method (Chapter 2), and specific fluorescence stains measured with the flow cytometry method and the direct count method (Wattanaphansak et al., 2005). Of these, the animal models are the most expensive procedures and the results are often influenced by several factors (e.g. pig immunity, variety of incubation periods form 7 to 14 days, and the proportion of dead and live bacteria). The cell culture systems and the fluorescence staining offer cheaper, faster, and more reproducible assays to measure *L. intracellularis* viability.

In the present study, the modified tissue culture method and the direct count method were applied to evaluate the effectiveness of a powder disinfectant, Stalosan® F, against *L. intracellularis*. The results obtained from the modified tissue culture method rely on the number of HICs, which is a relative percentage of bacteria that survive and remain active to infect the enterocyte (McOrist et al., 1995c; Collins et al., 2000; Chapter 2). Unlike the modified tissue culture assay, the direct count method measures the viability status of *L. intracellularis* based on the membrane integrity of the bacteria. 'Dead' bacterial populations are stained with red fluorescence of propidium iodine (PI). In contrast, healthy bacteria with intact cell membranes classified as 'live' populations are stained with green fluorescence of SYTO-9, resulting in the difference between live and dead bacterial populations.

The results from the modified tissue culture method and direct count method in determining the effectiveness of Stalosan® F were similar. Both assays showed that the reduction of *L. intracellularis* viability depended on dose and exposure time. For the

powder form, no viable *L. intracellularis* was found after exposure to all tested powder concentrations for 30 min as demonstrated with the modified tissue culture method. However, a few viable *L. intracellularis* organisms (<1%), as indicated by green fluorescent bacteria, were found when measured with the direct count method. The surviving population of *L. intracellularis* was reduced when the concentration of suspension and exposure time were increased for the suspension form. The modified tissue culture method showed that no viable *L. intracellularis* was found after exposure to an ≥8% concentration of the suspension for 30 min. Similarly, the direct count method showed that viable *L. intracellularis* was reduced to <1% after exposure to the suspension at concentrations of ≥16%. Stalosan® F was formulated according to the recommended use as a powder. However, the suspension form was also tested with the intention of simulating the effect of the powder mixing with the pig's drinking water or urine when applied on the floor. Furthermore, to separate the bacteria from the powder, an aqueous solution had to be mixed with the powder. The results from both assays indicated that either the powder or suspension form of Stalosan® F could be used for *L. intracellularis* inactivation.

After exposure to the powder and suspension forms of Stalosan® F, a few viable *L. intracellularis* (<1%) were detected as shown in the direct count method results, while none of the viable bacteria were found when measured with the modified tissue culture. In this situation it is possible that the green-labeled *L. intracellularis* in the direct count method (which were believed to be live) might have been in the viable but nonculturable (VBNC) state. The bacteria in this state generally remain viable and capable of revival under favorable conditions, but the standard culture methods cannot

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detect the bacteria. Many Gram-negative bacteria such as *Escherichia coli* (Xu et al., 1982), *Salomenlla enterica* serovar Enteritidis and serovar Typhimurium (Roszak et al., 1984; Gupte et al., 2003), and *Campylobacter jejuni* (Tholozan et al., 1999) employ this stage in order to survive in poor environmental conditions. However, the state of VBNC for *L. intracellularis* has not been reported and has not been completely investigated in this study. In addition, Millard and Roth (1997) found that in the dead bacteria with minimal or partial membrane damage, PI showed limited penetration and accumulation in the cytoplasm of the bacteria. Therefore, it is plausible that the green fluorescent bacteria are dead *L. intracellularis* with minimal membrane damage causing a greater accumulation of SYTO 9 than PI. Clearly, the concentrations of bacteria dramatically decreased after exposure to both forms of Stalosan® F. To date, although the mechanism of action of Stalosan® F remains unclear, it is possible that the membrane of the bacteria was destroyed by Stalosan® F. The abnormality of *L. intracellularis* membranes was observed using the SEM, as some parts of the bacterial membrane were clear and transparent after treatment.

In this study, the limitations of the modified tissue culture are that the viable or non-viable status of bacteria could not be directly differentiated in a mixed bacterial population. Furthermore, since the nature of *L. intracellularis* is a slow growth bacterium, the definitive results of the inoculum viability were not available until after 5 days of incubations. Although both the modified tissue culture and conventional tissue culture methods required the same incubation period to allow for bacterial growth, the modified tissue culture enabled the testing of several disinfectants, a variety of disinfectant concentrations, and several exposure times at the same time. In addition,

the assay was faster since the samples were tested as a batch on a 96-well tissue culture plate, while the conventional tissue culture operates the samples as individuals (Collins et al., 2000).

The advantages of the direct count method are the simplicity of the protocol and the ability to estimate the viability of bacteria directly from the whole population. Furthermore, the assay can be performed from start to finish and the data can be analyzed within 4 to 5 hours. However, the limitations of the direct count method are that this technique requires observation of the whole cell of bacteria stained with red or green fluorescence for counting. Therefore, if the direct count method is applied to determine the effectiveness of other disinfectants that lyse the whole cell of the bacteria, the interpretations of the results may be inconclusive. Moreover, the direct count method requires the use of an expensive fluorescent microscope to visualize the bacteria.

In summary, it has been demonstrated that the modified tissue culture method and direct count method give similar results for measuring the viability status of *L. intracellularis* after exposure to a powder disinfectant. Our results indicate that Stalosan® F in both a powder concentration of $\geq 0.25$ g/cm$^2$ and an aqueous suspension of $\geq 16\%$ concentration is able to inactivate over 99% of both *L. intracellularis* strains after 30 min of exposure as determined by both tests.

## 3.5 Acknowledgements
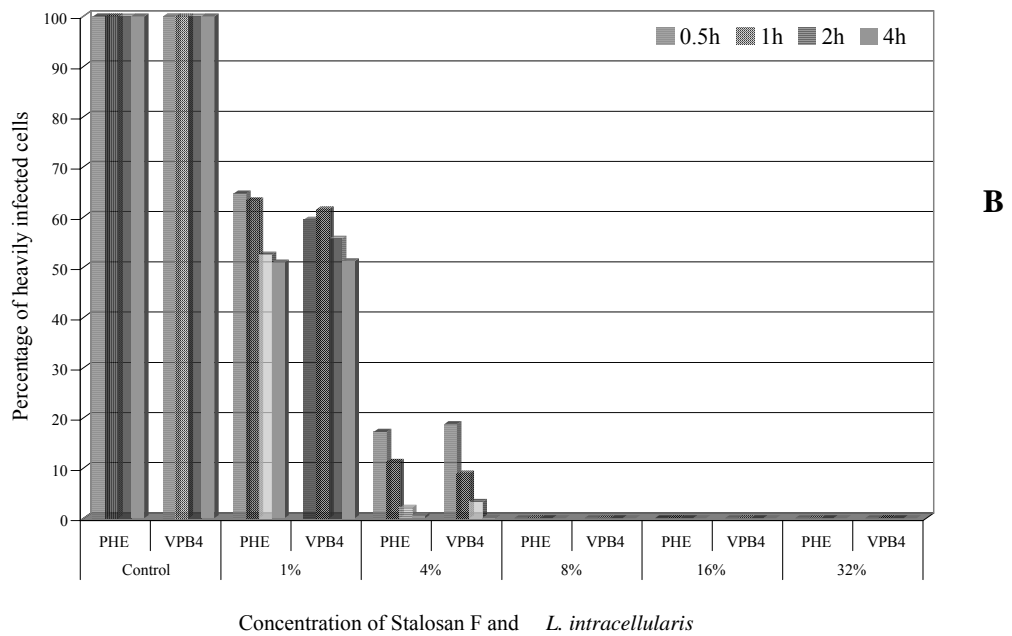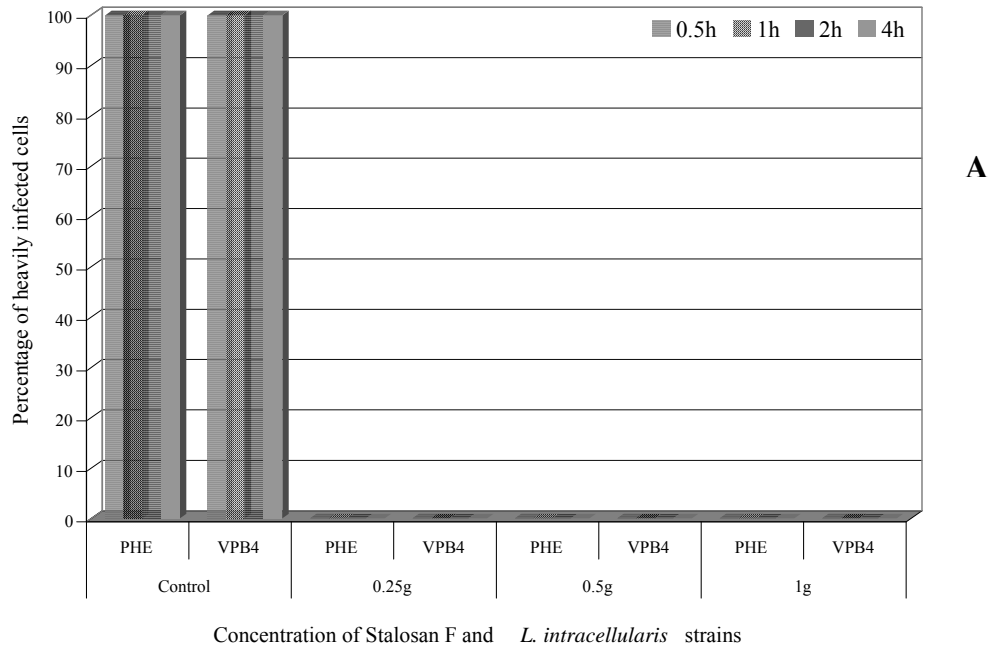
**Figure 3.1** The effectiveness of Stalosan® F used as a powder (A) and as an aqueous suspension (B) against *L. intracellularis* measured with the modified tissue culture method.
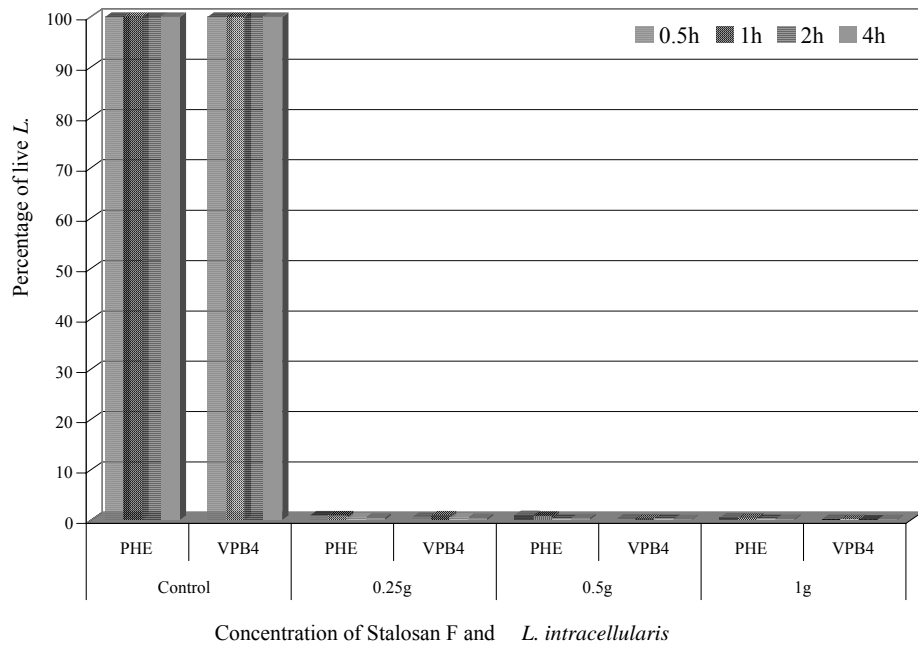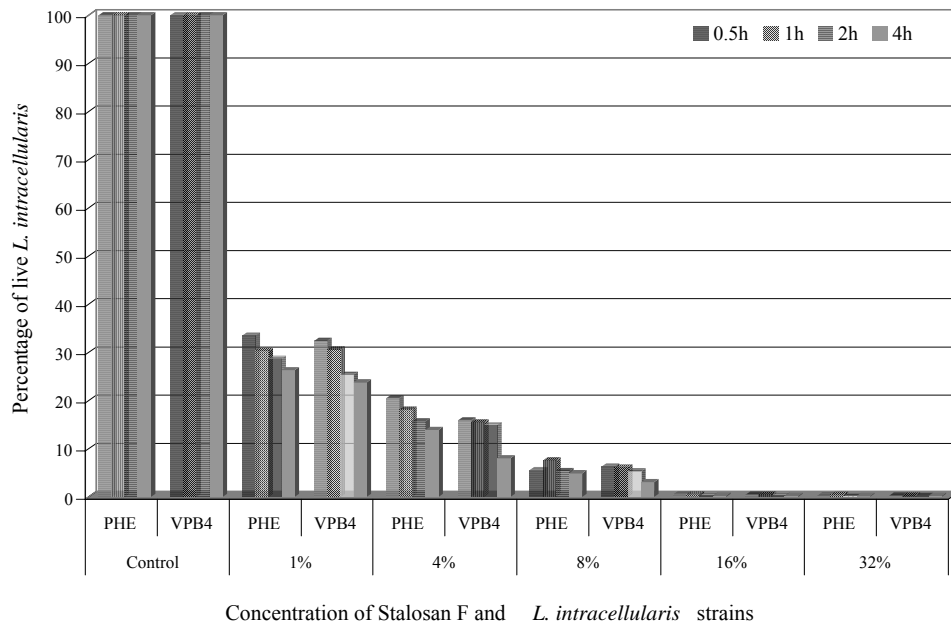
**Figure 3.2** The effectiveness of Stalosan® F used as a powder (A) and as an aqueous suspension (B) against *L. intracellularis* measured with the direct count method.
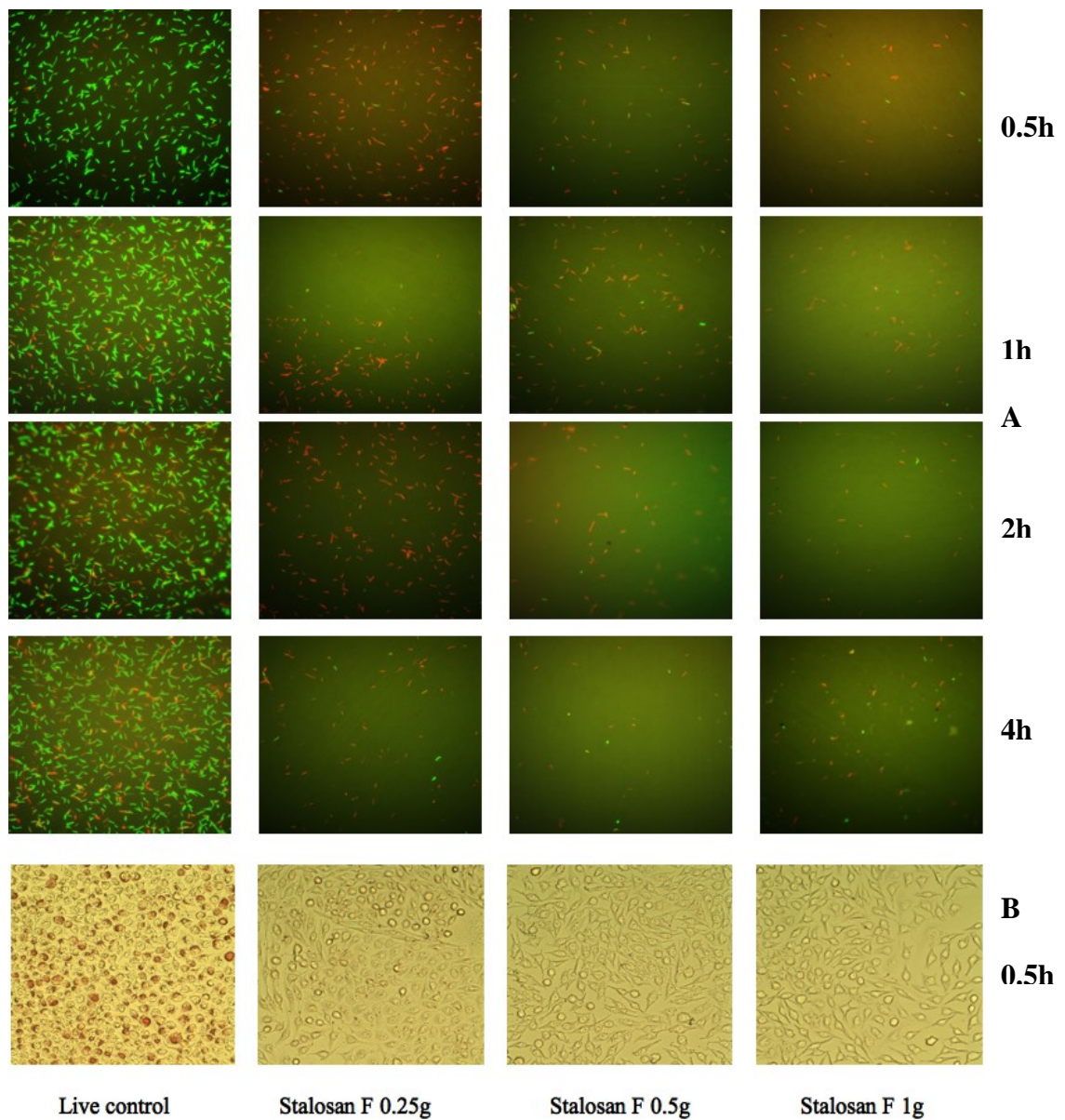
**Figure 3.3** *L. intracellularis* exposed to Stalosan® F powder at 0.25g, 0.5g, and 1g for 0.5h, 1h, 2h, and 4h. The viability of *L. intracellularis* was measured with the direct count method (A) and the modified tissue culture method (B).
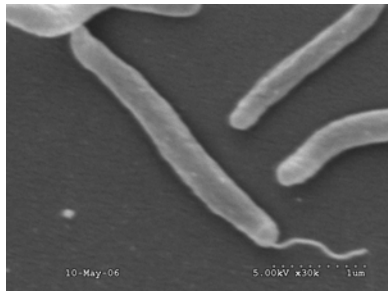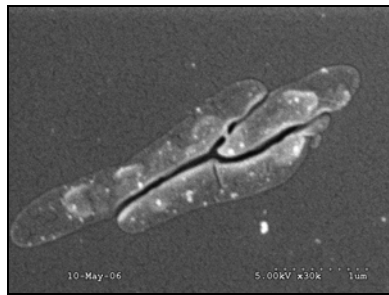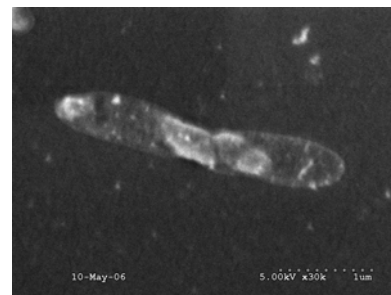
**Figure 3.4** Scanning electron micrograph of normal *L. intracellularis* (A) and after exposure to 4% (B), 16% (C) aqueous suspension, and 0.5 g of dry powder (D) Stalosan® F.

CHAPTER 4

EVALUATION OF *IN VITRO* BACTERICIDAL ACTIVITY OF COMMERCIAL

DISINFECTANTS AGAINST *LAWSONIA INTRACELLULARIS*

Submitted to JSHAP

*Lawsonia intracellularis* is an obligate intracellular bacterium causing of proliferative enteropathy in pigs, horses, and other species. Little information is available about the effectiveness of disinfectants against *L. intracellularis*. The objective of this study was to evaluate the bactericidal activity of 7 commercial disinfectants against *L. intracellularis* using a tissue culture system. Two *L. intracellularis* isolates were tested for susceptibility to Certi-Dine[®], DC&R[®], Nolvasan[®] S, Roccal[®]-D, Synergize[®], Tek-Trol[®], and Virkon[®]-S. All disinfectants were diluted with synthetic water containing 400 or 1000 ppm of calcium carbonate to test the influence of water hardness and 5% fetal bovine serum as organic material. The effects of disinfectant concentrations (0.5x, 1x, and 2x the recommended dose) and exposure times (10, 30, and 60 min) on each concentration were investigated. The number of cells heavily infected with *L. intracellularis* were counted and used as the *L. intracellularis* viability indicator to be compared with disinfectant-free controls. The susceptibilities of both *L. intracellularis* isolates to the disinfectants were similar. When recommended doses of disinfectants were tested for 10 min at 400 ppm synthetic water hardness, both *L. intracellularis* isolates were completely inactivated with DC&R[®] (1:128), Roccal[®]-D (1:256), and Synergize[®] (1:256). Inactivation was ≥ 99% with Virkon[®]-S (1%), 90-99% with Nolvasan[®] S (1:128) and Tek-Trol[®] (1:256), and <90% with Certi-Dine[®] (1:128). When the synthetic water hardness was increased to 1000 ppm, only the effectiveness of Synergize[®] was unchanged; the efficacies of the other disinfectants were reduced slightly. These data can serve as an *in vitro* guide for disinfectant selection in the control of *L. intracellularis*.

**4.1 Introduction**

*Lawsonia intracellularis* is an important enteric pathogen responsible for causing proliferative enteropathy (PE) in pigs, horses, and other species. The disease is economically important in pigs and mainly affects growing-finishing pigs with a variety of clinical signs. The acute form of PE in pigs manifests itself as bloody diarrhea, often with sudden death, and occurs mainly in mature pigs. The chronic form is often found in younger growing pigs and is characterized by chronic diarrhea and poor growth rates. The subclinical form results in slow growth without diarrhea (Lawson and Gebhart, 2000). Annual loss attributed to PE is estimated to exceed tens of millions of dollars for the United States swine industry alone (Winkelman, 1996a). A National Animal Health Monitoring System (NAHMS) serological survey reported that 96% of swine herds are seropositive for *L. intracellularis* (Bane et al., 1997). Furthermore, the with-in herd seroprevalence ranges from 11% to 92% in growing herds (Marsteller et al., 2003), indicating high disease prevalence throughout the United States' entire swine industry.

The mechanism of disease spread in herds is not fully understood, though infection among pigs is mainly transmitted through a fecal-oral route. Infected pigs can continuously shed the organism through feces and be a source of infection for up to 10 weeks, and the amount of bacteria shed has been estimated to be as high as $7 \times 10^8$ *L. intracellularis* per gram of feces (Smith and McOrist, 1997). Shed bacteria can remain viable and infective in pig feces for 2 weeks (Collins et al., 2000). Therefore, the horizontal transmission of PE among new, susceptible pigs can easily occur as a continuous infectious cycle via fecal material and fomites.

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To control PE outbreaks and transmission in swine herds, proper disinfection of housing, pens, and equipment is important to eliminate or reduce the number of active *L. intracellularis* in the environment. However, little information is available on the efficacy of various disinfectants against *L. intracellularis*. This is largely due to the fact that *L. intracellularis* is an obligately intracellular bacterium that only propagates itself inside the host cell. Thus it is difficult to perform primary bacterial isolation or find a reliable and reproducible intracellular assay to measure the efficacy of disinfectants against *L. intracellularis* as well as other obligately intracellular organisms.

To date, only two *in vitro* studies report bactericidal activity of some disinfectants against *L. intracellularis*; one uses a conventional tissue culture method (Collins et al., 2000) and the other used a modified tissue culture method as well as a direct count method with specific fluorescent staining (Chapter 3). In this current modified tissue culture method a large number of disinfectants over various concentrations and exposure times can be tested simultaneously. Since the results obtained from the modified tissue culture method are similar to the direct count method, either assay is appropriate for determining the activity of disinfectants against *L. intracellularis* (Chapter 3).

The objective of this study was to use a modified tissue culture method to evaluate the *in vitro* bactericidal activity of 7 commercial disinfectants commonly used on swine farms. The *in vitro* conditions were reproduced by the addition of 2 levels of water hardness and organic material to the assays. The disinfectants used in this study were selected to represent several classes of active ingredients, including iodine, quaternary ammonium compounds, aldehyde, phenol, biguanides, and oxidizing agents.

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Furthermore, the morphology of *L. intracellularis* after exposure to these disinfectants was also investigated. Direct comparison of these results could provide information about the selection of disinfectants for use against *L. intracellularis*.

**4.2 Materials and methods**

**4.2.1 Microorganism preparations**

Two *L. intracellularis* isolates were used in this study, strains PHE/MN1-00 and NWumn05, obtained and isolated from affected pigs in the U.S. in 2000 and 2005, respectively. Both isolates were stored at $-72^{o}C$ until use. Each isolate was grown for 3 passages in murine fibroblast-like McCoy cells (CRL 1696, American Type Culture Collection, Virginia, USA) to allow recovery from the frozen stage and to obtain 100% confluence of viable bacteria in the cell cultures. The bacteria were grown and harvested as described elsewhere (Guedes and Gebhart, 2003a; Wattanaphansak et al., 2005). Each isolate of *L. intracellularis* was tested twice and each replicate of bacterial preparation was independently prepared from 10 T-175 tissue culture flasks. The final concentration of each preparation was measured using the direct count method with immunostaining as previously described (Guedes and Gebhart, 2003a; Chapter 2).

### 4.2.2 Disinfectants

Seven commercial disinfectants were selected to represent several classes of products commonly used in the swine industry. The commercial disinfectants, their main active ingredients, and the recommended-use concentrations are summarized in Table 4.1. To compare the bactericidal activity of the disinfectants, Certi-Dine[®] (Certified Safety Manufacturing Inc.), DC&R[®] (Neogen), Nolvasan[®] S (Fort Dodge Laboratories), Roccal[®]-D (Pfizer Animal Health), Tek-Trol[®](Bio-Tek Industries), Virkon[®]-S (Antec International), and Synergize[®] (Preserve International) were prepared to final concentrations of 0.5x, 1x, and 2x, where x is the manufacturer's label instructions (Table 4.1).

### 4.2.3 Test procedures

All disinfectants were diluted with synthetic water containing 400 ppm or 1000 ppm of calcium carbonate ($CaCO_3$) to test the influence of water hardness on their effectiveness. Both concentrations of synthetic water were freshly prepared as described elsewhere (Association of Official Analytical Chemists (A.O.A.C), 1980). 5% fetal bovine serum (FBS) was added to each concentration of synthetic water to represent the presence of organic material. The final pH of the synthetic water used in this study was adjusted to 7.6.

The working solutions of each disinfectant were freshly prepared to final concentrations of 0.5x, 1x, and 2x. Eight ml of each disinfectant concentration were aliquoted into 15-ml polypropylene tubes, and 300 µl of *L. intracellularis* solution containing approximately $10^8$ *L. intracellularis*/ml were added to each tube and mixed.

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The bacterial suspensions were then filtered through 5-μm filters and transferred to 2-ml microcentrifuge tubes. The tubes were incubated at room temperature (22 to 25$^{\circ}$C) for 10, 30, or 60 min. After incubation, the bacterial suspensions were centrifuged at 10,000 rpm for 3 min. The pellets were washed and resuspended 3 times with 1.8 ml of the combination solution of Dulbecco's Modified Eagles Medium (DMEM) and 30% fetal bovine serum (FBS). After the final wash, the pellets were resuspended with 1 ml of tissue culture media (DMEM + 20% FBS + 0.5% Amphotericin B + 1% L-glutamine) for tissue culture infection. The controls used at each time point were live *L. intracellularis* in synthetic water without exposure to disinfectant and dead *L. intracellularis* in which the bacteria were inactivated using isopropyl alcohol for 30 min (Wattanaphansak et al., 2005). In this study, each concentration of the tested disinfectant was evaluated in duplicate, and both strains of *L. intracellularis* were tested twice in 400 and 1000 ppm concentrations of synthetic water.

The effectiveness of the disinfectants against *L. intracellularis* was evaluated using bacterial viability after exposure as the indicator. The viability of *L. intracellularis* was measured using a modified tissue culture method in which the bacteria were grown in 96-well tissue culture plates as previously described (Chapter 2 and 3). Briefly, after treatment with disinfectant, one hundred μl of the bacterial suspension were transferred to infect 2-day-old McCoy cells in quadruplicate cultures in 96-well tissue culture plates. After 24 hours of incubation, the culture medium was changed every day for 3 consecutive days. After 5 days of total incubation in an atmosphere of 8% oxygen, 8.8% carbon dioxide, and 83.3% nitrogen, the infected plates were harvested and fixed with a 50:50 mixture of acetone:methanol. Fixed plates were then stained with the modified

immunoperoxidase monolayer assay (IPMA) procedure using rabbit polyclonal as previously described (Chapter 2) and the number of heavily infected cells (HICs) was assessed. Cells were considered to be heavily infected if the number of *L. intracellularis* inside the cells exceeded 30 organisms (McOrist et al., 1995c). The number of HICs were counted and used as the *L. intracellularis* viability indicator as compared to the disinfectant-free controls.

### 4.2.4 Scanning electron microscopy (SEM)

To observe the bacterial morphology after exposure to the disinfectants, *L. intracellularis* was exposed to a 1x concentration of each tested disinfectant for 10 min. The bacterial suspensions were then processed for examination as described previously (Chapter 3) using a VPSEM-Hitachi S-3500N scanning electron microscope.

### 4.2.5 Data analysis

The HIC count for each time point and disinfectant concentration was expressed as the percent by which the bacteria concentration was reduced relative to the untreated control samples. The percentage of *L. intracellularis* surviving for each time, concentration, and disinfectant was averaged across the two individual tests.

**4.3 Results**

The *in vitro* susceptibilities of each *L. intracellularis* isolate to the 7 disinfectants are summarized in Figure 4.1 and Table 4.2. The results obtained from both *L. intracellularis* isolates were similar with respect to their susceptibilities to disinfectants at the label-recommended concentrations (1x). When the disinfectants were diluted with 400 ppm of water hardness and 5% organic material, both *L. intracellularis* isolates were completely inactivated within 10 min using Synergize® (1:256), Roccal®-D (1:256), and DC&R® (1:128). Within 10 min of exposure, Virkon®-S (1%) inactivated ≥ 99% of bacteria, Tek-Trol® (1:256) and Nolvasan® S (1:128) inactivated 90-99% of bacteria, and Certi-Dine® (1:128) inactivated < 90% of bacteria. The surviving population of each *L. intracellularis* isolate was decreased when the disinfectant exposure time increased from 10 min to 60 min and the disinfectant concentration increased from 0.5x to 2x (Figures 4.1A and 4.1C).

As the hardness of the synthetic water was increased to 1000 ppm of $CaCO_3$, the efficacies of most of the tested disinfectants were reduced. However, the effectiveness of Synergize® was unchanged and the effectiveness of Roccal®-D (1:256), DC&R® (1:128), and Virkon®-S (1%) were that of ≥ 99% inactivation. The effectiveness of the remaining disinfectants decreased slightly more (Figures 4.1B and 4.1D).

The morphology of *L. intracellularis* was investigated after the bacteria were exposed to the recommended concentration of each disinfectant for 10 min (Figure 4.2). Scanning electron microscopy showed that most bacterial populations disappeared when treated with Synergize®, Roccal®-D, and Virkon®-S. Only the particle-like, ruptured *L. intracellularis* cell membrane was found in each field of the electron

microscope. In contrast, when bacteria were treated with Certi-Dine®, DC&R®, Nolvasan® S, and Tek-Trol®, the density of the bacterial population and the bacterial morphology, such as cell shape, size, flagellum, and membrane structure, were similar to those found in the control.

## 4.4 Discussion

Although the mechanism of PE transmission among pigs and other animal species is not fully understood, it seems that bacterial contamination from feces and the environment play an important role. Antibiotics to which *L. intracellularis* is susceptible only eliminate the organism from inside animals, while disinfectants only inactivate *L. intracellularis* in the environment. Therefore, *L. intracellularis* from both the animal and the environment should be eliminated and inactivated to reduce the potential for disease spread.

In this study, seven commercially available disinfectants commonly used in swine facilities were evaluated for their bactericidal activity against 2 U.S. isolates of *L. intracellularis*. The modified tissue culture technique validated in a previous study (Chapter 3) was used because the assay makes it possible to simultaneously test several disinfectants across multiple concentrations and exposure times. The assay results are then valid for side-by-side comparisons. Moreover, our preliminary results showed that once *L. intracellularis* was exposed to formalin, the use of the direct count method with fluorescence staining could not differentiate between live and dead bacteria; all bacteria, both live and dead, were stained with green fluorescence (data not shown). Since a formaldehyde-based disinfectant (DC&R®) was included in this study, the use of the

tissue culture system was more appropriate to determine the bactericidal activity of this disinfectant.

In the U.S., water hardness levels vary across geographical regions and have been reported to range from 250 to 2848 ppm of $CaCO_3$ in some areas. Water hardness is considered to be at a very high level when $CaCO_3$ exceeds 300 ppm (American Water Works Association, 2003). More importantly, disinfectants are commonly used in environments containing organic materials such as feces, soil, and blood. Therefore, this study tested the disinfectants' activities against *L. intracellularis* in mimicked field conditions. Of the disinfectants evaluated, Synergize®, Roccal®-D, DC&R®, and Virkon®-S, applied at recommended concentrations, were the most active against *L. intracellularis in vitro*. All showed ≥99% inactivation of both *L. intracellularis* isolates in the presence of 400 or 1000 ppm of $CaCO_3$ and 5% organic material after 10 min of exposure. However, when the concentration of $CaCO_3$ in synthetic water increased from 400 to 1000 ppm, the bactericidal activities of these disinfectants were slightly decreased. Only Synergize® remained 100% effective against both isolates of *L. intracellularis*.

The active ingredients of some tested disinfectants were a mixture of several compounds. For instance, Synergize® is a combination of glutaraldehyde and quaternary ammonium compounds (QAC), and DC&R® is a combination of formaldehyde and QAC. Therefore, it was beyond the scope of this study to determine the mechanisms by which the disinfectants inactivated *L. intracellularis*. Morphological analysis of disinfectant-treated bacteria indicated particle-like debris from bacterial membranes after treatments with Synergize®, Roccal®-D, and Virkon®-S. This indicates that these

disinfectants might have the potential to lyse *L. intracellularis* cells. Synergize[®] and Roccal[®]-D are composed of QAC which have membrane-active agents that cause cell wall lysis by autolytic enzymes (McDonnell and Russell, 1999). Alternatively, Virkon[®]-S is a combination of peroxygen compounds that presumably denature essential proteins and cell wall structure (McDonnell and Russell, 1999). Like our study, El-Naggar et al. (2001) found that the morphological structure of *E. coli* exhibited lysation after exposure to 0.25% Virkon[®]-S for 15 min. However, no notable change in *E. coli* morphology was observed upon treatment with 0.03% Virkon[®]-S for 60 min. The morphology of the formaldehyde-treated *L. intracellularis* appeared intact, as with the control. These findings were similar to the report of El-Naggar et al. (2001), which found that formalin-treated *E. coli* display morphologic characteristics similar to the control.

In a previous *in vitro* study, Collins et al. (2000) reported that *L. intracellularis* was highly inactivated by 3.3% cetrimide (QAC) and 1% povidone-iodine, while the bacteria tolerated 30 min of exposure to a 0.33% phenolic compound and hydrogen peroxide/peracetic acid at a concentration of 0.0005%. There were differences in strain responses to 1% potassium peroxymonosulfate and 0.001% sodium hypochlorite. It is difficult to make direct comparisons between this study and previous investigations because of several factors. Major differences between the two studies include strains of *L. intracellularis* used (U.S. versus United Kingdom origin), bacterial concentrations ($10^8$ versus $10^4$ *L. intracellularis*/ml), testing concentrations, and exposure times. Furthermore, results from the previous study were obtained without simulation of water hardness or organic material load. However, there were notable similarities among the

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results. As in the previous study, both *L. intracellularis* U.S. isolates in this study were highly susceptible to QAC-based disinfectants and potassium peroxymonosulfate and tolerated the phenolic mixtures. In contrast, povidone-iodine-based disinfectants showed less effectiveness against *L. intracellularis*. The decreased bactericidal activity of povidone-iodine is mainly attributable to the effects of organic material in the synthetic water. However, when the concentration of povidone-iodine was increased to 2x, the percentages of viable *L. intracellularis* were dramatically reduced. Moore and Payne (2004) described that organic material has few effects in the presence of high iodine concentrations, whereas low iodine concentrations are more influenced by organic matter.

Our results showed that both *L. intracellularis* isolates were similar in their susceptibility to disinfectants, while a previous study showed differences in strain responses to sodium hypochlorite and potassium peroxymonosulfate (Collins et al., 2000). However, the limited number of strains used in each study makes it difficult to draw any conclusions about the differing responses to disinfectant types among strains. The authors are unaware of any in-depth investigations about *L. intracellularis* tolerance to disinfectants. This warrants further investigation since several studies have reported that bacteria resistant to disinfectants have the potential to develop resistance to some antibiotics (Russell, 2001; Sidhu et al., 2002). Russell (2001) and Thomas et al. (2005) described that the incorrect use of disinfectants or their use with sub-lethal dosages could possibly generate bacterial populations resistant to disinfectants.

In this investigation, it was clear that the disinfectants' bactericidal activities against *L. intracellularis* were dependent upon concentrations, exposure times, and

water hardness levels. The results showed that when the disinfectant concentrations were increased from 0.5x to 2x label dosage, the bactericidal activities of all disinfectants increased with reduced exposure time. Several investigations have reported similar dose-response relationships where higher disinfectant concentrations required shorter exposure times to inactivate organisms (El-Naggar et al., 2001; Moretro et al., 2009; Chapter 3). Although increasing the concentrations of disinfectants increased bactericidal activity, this over-application has several disadvantages, including expense, increased toxicity to animals and laborers, and corrosion of metallic material. The 10-min exposure time was chosen for all disinfectants because it is the minimum exposure time that is recommended by most disinfectant manafacture for activity to occur. Our results also showed that the bactericidal activity of the disinfectants increased with prolonged exposure time. Therefore, when disinfectants are used under field conditions, extending the exposure time to the floor or equipment surface may also increase the effectiveness of the products.

Uncertainty remains as to the ability of an *in vitro* laboratory test in predicting a disinfectant's bactericidal activity on a swine farm. Most disinfectant investigations, including this study, have tested bactericidal activity against microorganisms under suspension conditions. In reality, it is difficult to predict a disinfectant's efficacy against sessile bacteria located on a dry, dirty surface. Several investigations have reported that the bactericidal activities of disinfectants tested against bacteria on dry surfaces are lower than those in suspension, although bacteria were completely inactivated when tested under suspension (Thomas et al., 2005; Moretro et al., 2009). Because several factors are involved in the efficacy of disinfectants under field conditions (including

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organic load, temperature, and water hardness), it is possible that the *in vitro* results of this study may not accurately predict disinfectant effectiveness in swine barns. Further evaluation of these disinfectants on dry surfaces or under field conditions is needed.

In conclusion, this investigation's side-by-side comparison of the *in vitro* activities of disinfectants against *L. intracellularis* could serve as a guide for disinfectant selection in the control of *L. intracellularis* in the environment. The results presented here expand upon the limited data available for *in vitro* disinfectant validation. The results of this study suggest that Synergize®, Roccal®-D, DC&R®, and Virkon®-S would perform well for the inactivation of *L. intracellularis*.

## 4.5 Acknowledgements

**Figure 4.1** The effectiveness of Synergize® (SYN), Roccal®-D (ROC), DC&R® (DCR) Virkon®-S (VIR), Tek-Trol® (TEK), Nolvasan® S (NOL), and Certi-Dine® (CER) against *L. intracellularis* strains PHE/MN1-00 (A and B) and NWumn05 (C and D) in the presence of $CaCO_3$ at 400 ppm (A and C) and 1000 ppm (B and D) with the combination of 5% fetal bovine serum (FBS). The bacteria were exposed to 0.5x, 1x, or 2x of each disinfectant's labeled dilution and incubated at room temperature for 10, 30, or 60 min before determining the bacterial viability using a modified tissue culture method.
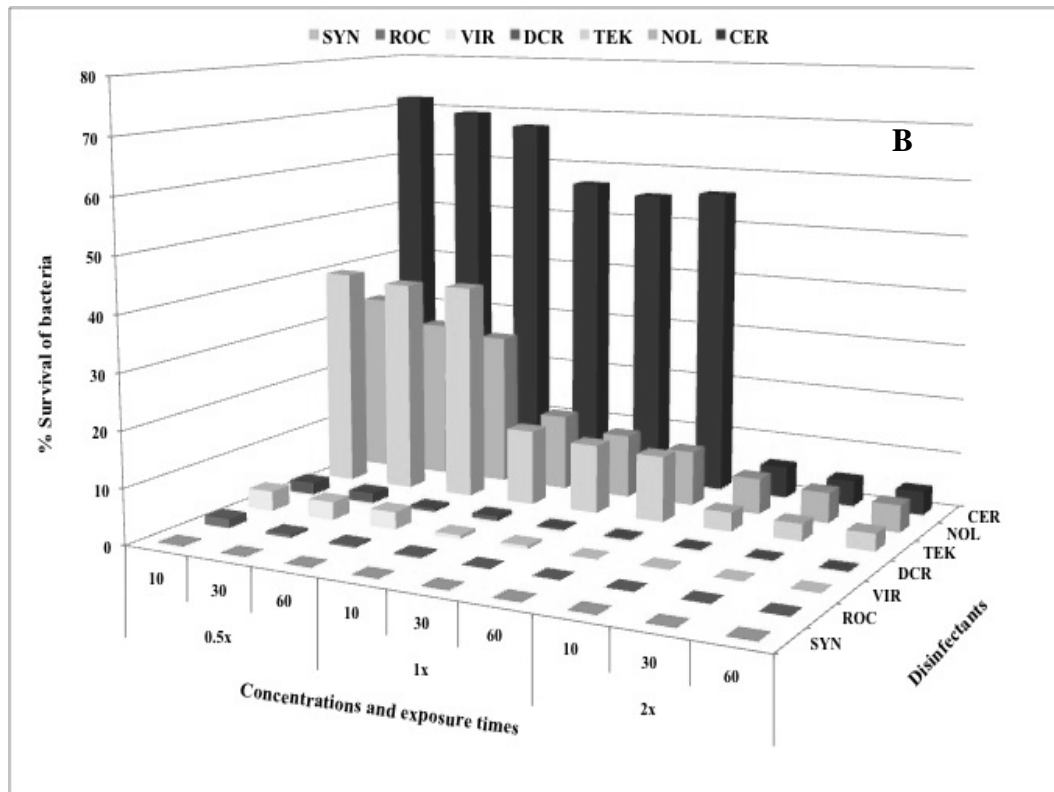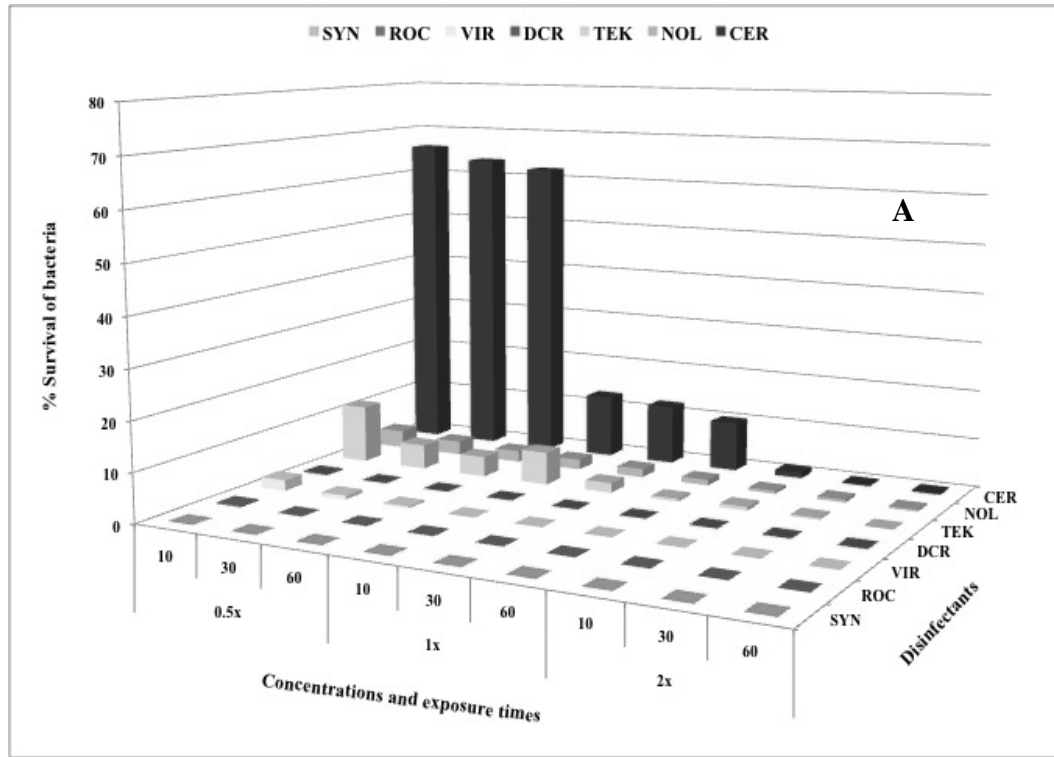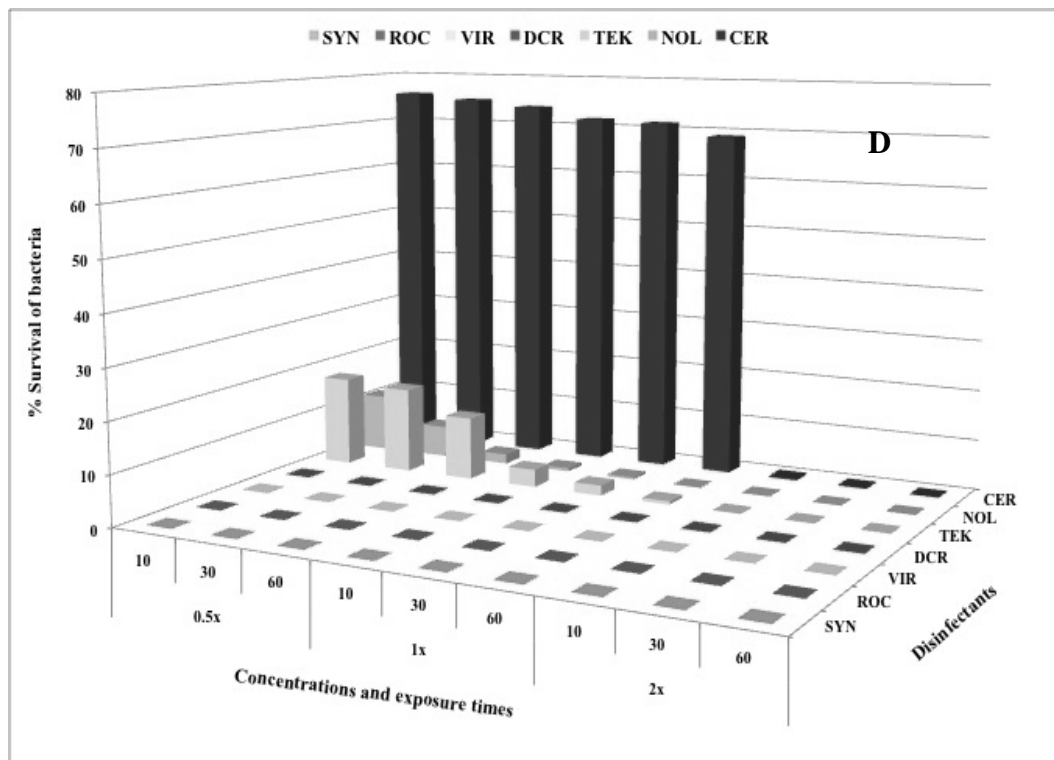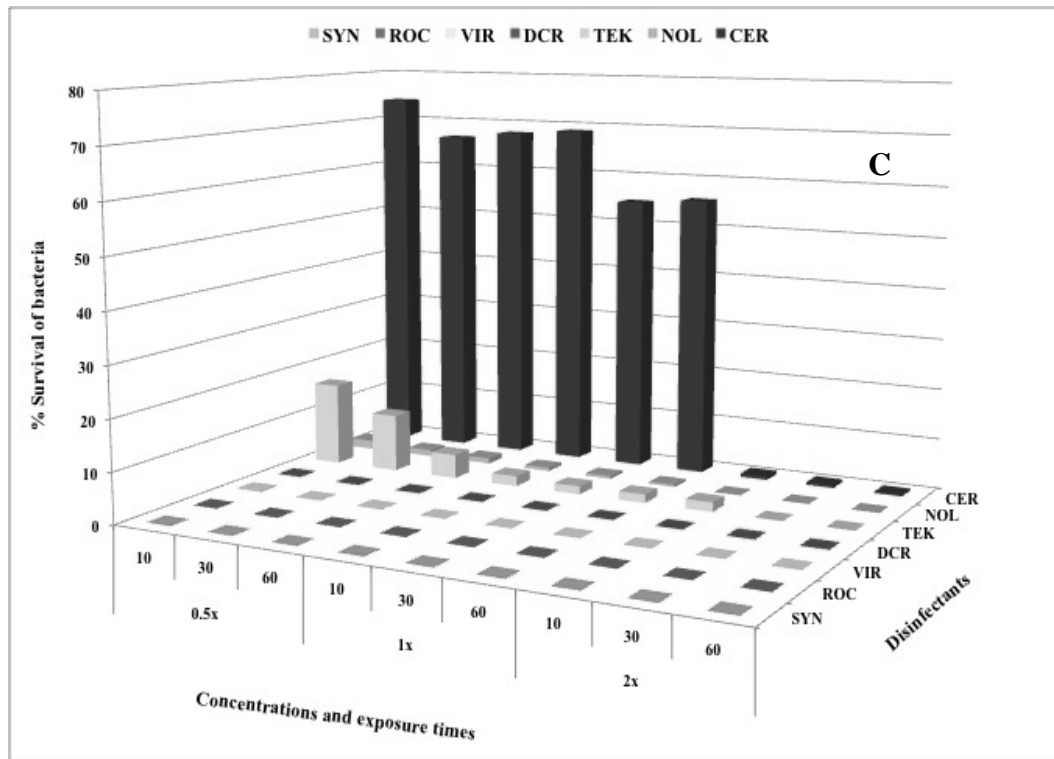
**Table 4.1** Summary of tested disinfectants and the recommended concentrations for use.

| Disinfectants | Class of disinfectants | Active ingredients | Recommended concentrations |
|---|---|---|---|
| Certi-Dine® | Iodine | Povidone–iodine 10% (1% available iodine) | 1:128 |
| DC&R® | Quaternary ammonium compounds Aldehyde | -2-(hydroxymethyl)-2-Nitro-1,3-Propanediol        19% <br><br>-Alky ($C_{12}$-67%; $C_{14}$-25%; $C_{16}$-7%; $C_8$, $C_{10}$, $C_{18}$-1%) dimethyl benzyl Ammonium chloride        3.08% <br>-Formaldehyde        2.28% | 1:128 |
| Nolvasan® S | Biguanides | 1,1'-Hexamethylenebis [5-(p-chlorophenyl) biguanide] diacetate (Chlorhexidine)        2% | 1:128 |
| Roccal®-D plus | Quaternary ammonium compounds | -Didecyl dimethyl ammonium chloride        9.2% <br><br>-Alkyl ($C_{12}$-61%; $C_{14}$-23%; $C_{18}$-25%; $C_8$,&$C_{10}$,-2.5%) dimethyl benzyl ammonium chloride        9.2% <br><br>-Alkyl ($C_{12}$-40%; $C_{14}$-50%; $C_{16}$-10%) dimethyl benzyl ammonium chloride        4.6% <br><br>-bis-n-tributyltin oxide        1.0% | 1:256 |

**Table 4.1** Continued.

| Disinfectants | Class of disinfectants | Active ingredients | | Recommended concentrations |
|---|---|---|---|---|
| Synergize® | Aldehyde Quaternary ammonium | -Glutaraldehyde | 7% | 1:256 |
| | | -Alkyl ($C_{12}$-67%; $C_{14}$-25%; $C_{16}$-7%; $C_{18}$-1%) dimethyl benzyl ammonium chloride | 26% | |
| Tek-Trol® | Phenol | -Ortho-Phenylphenol | 12% | 1:256 |
| | | -Ortho-Benzyl-para-chlorophenol | 10% | |
| | | -Oara-tertiary-Amylphenol | 4% | |
| Virkon®-S | Oxidizing agent | -Potassium peroxymonosulfate | 21% | 1% |
| | | -Sodium Chloride | 1.5% | |

**Table 4.2** Effects of tested disinfectants' concentrations and duration of exposure on the survival of *L. intracellularis* strain PHE/MN1-00 and NWumn05 under 5% FBS added in hard water at 400 ppm and 1000 pm conditions.

| Disinfectants | Concentration | % Survival of *L. intracellularis* PHE/MN1-00 | | | | | | % Survival of *L. intracellularis* Nwumn05 | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | 400 ppm $CaCO_3$ | | | 1000 ppm $CaCO_3$ | | | 400 ppm $CaCO_3$ | | | 1000 ppm $CaCO_3$ | | |
| | | Duration of exposure (min) | | | Duration of exposure (min) | | | Duration of exposure (min) | | | Duration of exposure (min) | | |
| | | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 |
| DC&R® | 0.5x | 0.24 | 0.04 | 0 | 2.17 | 1.79 | 0.57 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1x | 0 | 0 | 0 | 0.78 | 0.23 | 0.13 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2x | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Synergize® | 0.5x | 0.03 | 0 | 0 | 0.15 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1x | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2x | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Roccal®-D | 0.5x | 0.3 | 0 | 0 | 1.62 | 0.53 | 0.35 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1x | 0 | 0 | 0 | 0.29 | 0.06 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2x | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 4.2 Continued.**

| Disinfectants | Concentration | % Survival of *L. intracellularis* PHE/MN1-00 | | | | | | % Survival of *L. intracellularis* Nwumn05 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 400 ppm CaCO$_3$ | | | 1000 ppm CaCO$_3$ | | | 400 ppm CaCO$_3$ | | | 1000 ppm CaCO$_3$ | | |
| | | Duration of exposure (min) | | | Duration of exposure (min) | | | Duration of exposure (min) | | | Duration of exposure (min) | | |
| | | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 |
| Tek-Trol® | 0.5x | 11.80 | 4.99 | 4.08 | 39.81 | 38.74 | 39.18 | 16.35 | 11.38 | 4.76 | 19.74 | 18.76 | 13.82 |
| | 1x | 6.64 | 1.98 | 0.56 | 13.60 | 12.41 | 11.86 | 1.98 | 1.59 | 1.66 | 3.72 | 1.85 | 0.31 |
| | 2x | 0.71 | 0.37 | 0.03 | 3.39 | 3.09 | 3.11 | 1.85 | 0.01 | 0 | 0.02 | 0.03 | 0 |
| Virkon®-S | 0.5x | 2.17 | 0.76 | 0.36 | 3.55 | 3.20 | 3.05 | 0.18 | 0.10 | 0.07 | 0.04 | 0.03 | 0 |
| | 1x | 0.04 | 0.04 | 0 | 0.63 | 0.53 | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2x | 0 | 0 | 0 | 0.17 | 0.04 | 0.04 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nolvasan® S | 0.5x | 3.60 | 2.80 | 2.27 | 32.99 | 28.99 | 27.61 | 1.69 | 1.24 | 1.05 | 20.14 | 10.93 | 3.70 |
| | 1x | 2.04 | 1.72 | 1.13 | 13.73 | 11.47 | 9.93 | 0.65 | 0.53 | 0.43 | 0.48 | 0.14 | 0.11 |
| | 2x | 0.59 | 0.57 | 0.39 | 6.44 | 5.48 | 4.90 | 0 | 0 | 0 | 0.02 | 0.01 | 0 |
| Certi-Dine® | 0.5x | 65.97 | 63.48 | 62 | 71.89 | 69.28 | 67.17 | 74.08 | 66.51 | 67.83 | 75.16 | 74.64 | 74.03 |
| | 1x | 12.81 | 12.1 | 10.07 | 56.56 | 54.96 | 56.01 | 68.72 | 54.69 | 55.68 | 69.19 | 68.84 | 66.97 |
| | 2x | 1.16 | 0.27 | 0.29 | 5.66 | 4.64 | 4.22 | 0.68 | 0.53 | 0.32 | 0.34 | 0.17 | 0.08 |
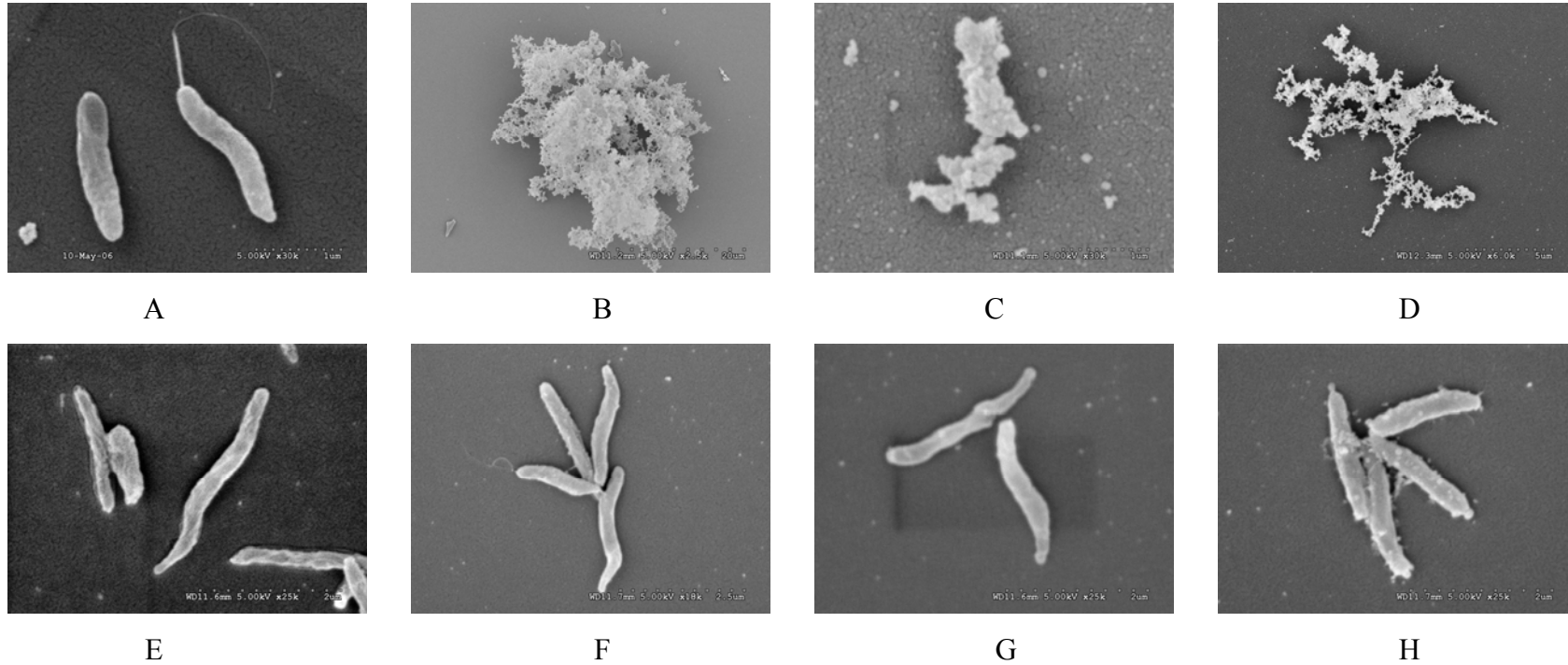
**Figure 4.2** Scanning electron microscopy showing the morphology of *L. intracellularis* strain NWumn05 after exposure to disinfectants for 10 min. (A) *L. intracellularis* was treated with phosphate buffered saline (control); cells were exposed to (B) Synergize® at a concentration of 1:256, (C) Roccal®-D at a concentration of 1:256, (D) Virkon®-S at a concentration of 1%, (E) DC&R® at a concentration of 1:128, (F) Tek-Trol® at a concentration of 1:256, (G) Certi-Dine® at a concentration of 1:128, and (H) Nolvasan® S at a concentration of 1:128.

SECTION III: PORCINE PROLIFERATIVE ENTEROPATHY –

QUANTITATIVE DIAGNOSIS

CHAPTER 5

DEVELOPMENT AND VALIDATION OF A POLYMERASE CHAIN

REACTION ASSAY FOR QUANTIFICATION OF *LAWSONIA*

*INTRACELLULARIS* IN SWINE FECES AND CELL CULTURE

Submitted to J. Vet. Diagn. Invest.

The objective of this study was to develop and validate a quantitative PCR (qPCR) assay using SYBR green for quantification of *Lawsonia intracellularis* in pure culture and pig fecal samples. Specific primers were designed and validated using the *aspA* gene as a target. Serial 10-fold dilutions of pure culture samples and several sets of spiked feces containing approximately $10^8$ to $10^1$ *L. intracellularis*/ml and *L. intracellularis*/g of feces, respectively, were used for qPCR validation. In pure culture, the limit of detection and quantification of *L. intracellularis* was $5.1 \times 10^1$ *L. intracellularis*/ml while the lower limit of the linear range of the assay in pure culture was $5.1 \times 10^2$ *L. intracellularis*/ml. The limit of detection of *L. intracellularis* in spiked feces was $2.55 \times 10^1$ *L. intracellularis*/g while the limit of quantification was $2.55 \times 10^3$ *L. intracellularis*/g. A concentration between $2.55 \times 10^4$ and $2.55 \times 10^3$ *L. intracellularis*/g was the lower limit of the linear range. No cross-reactivity of qPCR was found when the assay was tested using the DNA extracted from 16 species of enteric bacteria that are commonly found in pigs or closely related to *L. intracellularis*. The new qPCR assay is a sensitive, specific, precise, and accurate method for the detection and quantification of *L. intracellularis* in samples.

**5.1 Introduction**

*Lawsonia intracellularis*, a Gram-negative and obligately intracellular bacterium, is the causative agent of proliferative enteropathy (PE) in swine and other animal species. The typical lesion of PE in pigs is the thickening of the intestinal mucosa in predominantly the small intestine, especially the ileum (Lawson and Gebhart, 2000). The disease is mainly transmitted among pigs through a fecal-oral route. It has been found that the bacteria shed in feces can maintain infectivity outside of their host for 2 weeks (Collins et al., 2000). Therefore, fecal material is considered an important source for horizontal disease transmission.

Little is known about the dynamics of *L. intracellularis* fecal excretion post-infection. These limitations are attributable to a lack of quantitative assays for the accurate estimation of *L. intracellularis* in samples. Therefore, the peak of *L. intracellularis* excretion and quantity of fecal shedding remain poorly understood. For the qualitative detection of *L. intracellularis* in pig feces, previous experimental challenge studies have reported that inoculated pigs initially shed the bacteria 1 to 3 weeks post-inoculation (McOrist and Smith, 1997; Knittel et al., 1998; Guedes et al., 2002a; Guedes and Gebhart, 2003b). Additionally, infected pigs can shed the bacteria continuously for up to 10 to 12 weeks after infection (Smith and McOrist, 1997; Guedes and Gebhart, 2003b). These results were obtained through the use of conventional PCR, a qualitative assay that detects only the presence or absence of *L. intracellularis* DNA in feces. Although this molecular assay is widely accepted as a highly specific ante-mortem test and is able to detect as low as $2 \times 10^2$ *L. intracellularis*/gram of feces with nested PCR (Møller et al., 1998), its sensitivity is estimated to range between 40% and 70% due to the presence of fecal inhibitors (Knittel et al., 1998; Guedes et al., 2002d).

In some studies, the quantity or level of *L. intracellularis* infection in samples (pig feces, intestinal tissue, gut mucosal homogenate, and pure culture) is required. Previously, estimates of *L. intracellularis* loads in these samples were obtained using a cumbersome immunostaining assay with a monoclonal or polyclonal antibody specific for *L. intracellularis* applied on serial dilutions of the sample (Smith and McOrist, 1997; Guedes et al., 2002d; Guedes and Gebhart, 2003ab; Chapter 2). The stained bacteria were then manually counted under light or fluorescence microscopes. A previous experimental study that used these assays with fecal samples found that the amount of *L. intracellularis* fecal shedding from infected pigs was estimated to be approximately $5x10^4$ to $7x10^8$ *L. intracellularis*/g of feces (Smith and McOrist, 1997). Unfortunately, the limitations of the immunostaining assays on fecal samples include the time-consuming process, subjective results, and reduced diagnostic specificity.

Real-time quantitative PCR (qPCR) has been widely used for the quantification of bacteria in samples. Recently, at least two real-time PCR assays for *L. intracellularis* have been developed with their main purpose being the detection of *L. intracellularis* in fecal samples (Lindecrona et al., 2002; Pusterla et al., 2008). Another very recent study has validated a qPCR assay for the quantitative measurement of *L. intracellularis* in pig fecal samples (Nathues et al., 2009). All these studies were probe-based systems, which are highly specific but more expensive. Therefore, the overall purpose of this study was to develop and validate a new SYBR green qPCR assay for the detection and quantification of *L. intracellularis* in bacterial culture and pig feces.

## 5.2 Materials and methods

### 5.2.1 Bacterial strains and preparation

The *L. intracellularis* strain PHE/MN1-00 was used for all steps in the development and validation of the qPCR experiments. *L. intracellularis* pure culture passage between 140 and 150 was grown and harvested in 200 T-175 flasks as described previously (Guedes and Gebhart, 2003b; Wattanaphansak et al., 2005). The final concentrations of bacterial cultures were quantified using a direct count method as described previously (Guedes and Gebhart, 2003b; Chapter 2). Briefly, serial 10-fold dilutions of *L. intracellularis* suspensions were performed in phosphate buffered saline (PBS). Ten μl of each dilution was used to coat 15-well glass slides which were then incubated at $37^{o}C$ overnight. Six replicates were performed for each bacterial dilution. The slides were then fixed with cold acetone and stained with a modified immunoperoxidase assay using a rabbit polyclonal as previously described in Chapter 2. The numbers of red-stained bacteria were directly counted under a light microscope at a magnification of 630x from the dilutions that had approximately 50-500 *L. intracellularis*/well. The final concentration of *L. intracellularis* was estimated from the average of the 6 replicates of the counted dilution.

### 5.2.2 Preparation of *L. intracellularis* pure culture and spiked fecal samples

After determining the concentration of *L. intracellularis*, dilution series were created in pure culture and spiked feces. For the pure culture samples, serial 10-fold dilutions of bacteria were made in sterile PBS. The final concentrations of the bacterial dilutions ranged from $5.1x10^{8}$ to $5.1x10^{1}$ *L. intracellularis*/ml. For the *L.*

*intracellularis* spiked feces series, fecal samples from 5 individual pigs obtained from 4 PE-negative herds, each representing a different age group, were spiked with known *L. intracellularis* concentrations. All fecal samples were confirmed to be *L. intracellularis*-negative using a conventional PCR test (Jones et al., 1993ab). To make the spiked feces, Twenty ml of each pure culture dilution was added to 20g of *L. intracellularis*-negative feces and mixed thoroughly. Fecal sample 1 was spiked with bacterial concentrations of $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml, resulting in a set of feces spiked at concentrations of $2.55 \times 10^8$ to $2.55 \times 10^1$ *L. intracellularis*/g. In contrast, fecal samples 2, 3, 4, and 5 were spiked with *L. intracellularis* concentrations of $1.37 \times 10^5$, $1.37 \times 10^4$ and $1.37 \times 10^3$ *L. intracellularis*/ml to create low-load samples with final concentrations of $6.85 \times 10^4$, $6.85 \times 10^3$, and $6.85 \times 10^2$ *L. intracellularis*/g of feces, respectively. Pure culture and spiked feces 1 were used to estimate the limit of detection and accuracy of quantification. The low-load fecal samples were used to measure the accuracy and repeatability of qPCR at low *L. intracellularis* concentrations. Finally, all fecal samples were used to determine the effect of freezing and thawing on qPCR accuracy.

### 5.2.3 DNA extraction

From each concentration of *L. intracellularis* pure culture and spiked feces samples, three independent DNA extractions were performed using the methods described below. To extract *L. intracellularis* DNA from the fecal samples, two DNA extraction methods were compared. First, DNA was extracted using the QIAamp® DNA Stool Mini kit (QIAGEN, Valencia, CA, USA). The protocol was performed according to the manufacturer's instructions. Only the first DNA elution of 200 μl was used for qPCR analysis. Second, DNA was also extracted using the

protocol of Vanhoutte et al. (2004) with slight modification as described below. For this protocol, two-tenths of a gram (wet weight) of pig feces was homogenized in 1.8 ml of peptone buffer saline (1% peptone, 0.5% NaCl, 0.35% $Na_2HPO_4$, 0.15% $NaH_2PO_4$) and centrifuged at 16,000g for 5 min. After discarding the supernatant, the pellet was washed in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The pellet was re-suspended with 300 µl of lysozyme (40 mg/ml in TE) and incubated at $37^{o}C$ for 60 min. After incubation, seven hundred fifty µl of Guanidiumthiocyanate-EDTA-Sarkosyl solution (GES) (600 g/L Guanidium-thiocyanate, 0.1 M EDTA, 1% Sarkosyl) was added and vortexed. The suspension was then put on ice for 10 min. Three hundred fifty µl of 7.5 M ammonium acetate was added and put on ice continuously for 10 min. Next, five hundred µl of 24:1 chloroform/iso-amyl alcohol was added, mixed and centrifuged at 10,000g for 5 min to separate phases of suspension. After repeating the chloroform/iso-amyl alcohol extractions 3 times, one thousand µl of the upper aqueous layer was transferred to a new 2 ml tube and 540 µl (0.54 volume) of cold isopropyl alcohol was added and gently mixed. The suspension was incubated at room temperature for 10 min to allow the DNA to precipitate. After 5 min of centrifugation at 16,000g, the pellet was washed twice with 300 µl of 75% ethanol alcohol and allowed to dry at room temperature for 45 min. Finally, one hundred fifty µl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) was added to allow the DNA to re-suspend at $4^{o}C$ overnight. Later, the purified DNA solution was treated with 1 µl of 10 mg RNAse (Roche Applied Science, Indianapolis, IN, USA) and incubated at $37^{o}C$ for 90 min to remove the remaining RNA. The total DNA concentrations of all fecal sample

extractions were measured spectrophotometrically (Eppendorf North America, Westbury, NY, USA).

To extract *L. intracellularis* DNA from the pure culture samples, two DNA extraction methods were compared. First, the DNeasy® Blood and Tissue Kit (QIAGEN, USA) was used and the extraction was performed according to the manufacturer's instructions, with only the first 200 µl elution of DNA collected and used for qPCR analysis. Second, the DNA of *L. intracellularis* pure culture was extracted using the modified protocol of Vanhoutte et al. (2004) as described above, excluding the steps of washing the samples with peptone and TE buffer.

**5.2.4 qPCR primer design**

Six sets of primers were designed based on the *aspA* (aspartate ammonia-lyase) gene as a target using the Primer3 version 0.4.0 program (http://frodo.wi.mit.edu) (Rozen and Skaletsky, 2000). The sequence data of the *aspA* gene were obtained from http://www.ncbi.nlm.nih.gov. The main parameters for designing the primers included (1) product size ranges between 150 and 300 bp, (2) primer lengths between 18 and 27 nucleotides, (3) melting temperature (*Tm*) of the primers between 57 and 63$^\circ$C with 2$^\circ$C maximum *Tm* difference, (4) GC% content between 20 and 80%, (5) a GC clamp at the 3' end of both forward and reverse primers, (6) and no 3' complementary for primer pairs. The primers that amplified between nucleotide positions 911 and 1072 of the *L. intracellularis aspA* gene and generated an expected product of 162 bp were selected for use in this qPCR study. These primer set had the best efficiency and the tightest dissociation curve. The primers had the following sequences: F: 5'-GCTGTGGATTGGGAGAAATC-3'; R: 5'-CAAGTTGACCAGCCTCTGC-3'.

Hypothetical primer specificity was primarily confirmed by comparison with sequence data of other known organisms using the Basic Local Alignment Search Tool (BLAST) available at www.ncbi.nlm.nih.gov/BLAST/. To test the specificity and cross-reactivity of the selected primers, conventional PCR and qPCR assays were conducted with 16 pure cultures of the following reference bacterial strains: Hemolytic *Escherichia coli* ATCC 35218, *E. coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *S. typhimurium* (field isolate), *Shigella flexneri* ATCC 12022, *Campylobacter coli*, *C. jejuni*, *C. upsaliensis*, *Clostridium perfringens*, *Brachyspira hyodysenteriae*, *B. pilosicoli*, *B. innocens*, *Bacteroides fragilis*, *Yersinia enterocolitica* ATCC 9610, *Bilophila wadsworthia*, and *Desulfovibrio desulfuricans*. Furthermore, 20 negative control fecal samples obtained from a previous challenge study were also run with both assays to confirm the specificity of the selected primers. The DNA of the bacterial reference strains and *L. intracellularis*-negative pig fecal samples were extracted using the DNeasy® and the QIAamp® kits, respectively.

### 5.2.5 Conventional PCR and qPCR conditions

Conventional PCR was conducted in a final reaction volume of 25 μl using 5 μl of template DNA. The samples were amplified using an ABI® 2720 thermal cycler (Applied Biosystems Inc., Foster City, CA, USA) with the following conditions: 12.5 μl of HotStarTaq DNA Polymerase kit (QIAGEN, USA), 0.5 μl (0.6 μM) of each primer, 1 μl of 10 mg/ml bovine serum albumin (BSA), and 5.5 μl of ultra pure water. Cycling conditions were as follows: 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 60°C for 60 s and 72°C for 30 s. A final extension of

72°C was carried out for 7 min. Amplicons were separated on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

qPCR was conducted in a final reaction volume of 25 μl using 5 μl of template DNA. The qPCR mixture consisted of 12.5 μl of Brilliant® II SYBR® Green QPCR Master Mix (Stratagene-An Agilent Technologies Company, La Jolla, CA, USA), 0.5 μl (0.6 μM) of each primer, 0.375 μl of 1:500 ROX reference dye, 1 μl of 10 mg/ml BSA, and 5.125 μl of ultra pure water. Negative controls (non-template control (NTC) that contained the qPCR mixture but no DNA template) were added to each run. Additionally, serial 10-fold dilutions of purified *L. intracellularis* standard DNA (described below) were added in every run to generate the standard curves. All unknown samples, negative controls, and purified *L. intracellularis* standard DNA were tested in triplicate in the same run. The qPCR amplification and quantification were performed using an Mx3000P Real-Time PCR instrument (Stratagene-An Agilent Technologies Company, USA). The Thermal Profile of the qPCR reaction was set as follows: (Segment 1) initial denaturation at 95°C for 10 min, (Segment 2) 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 30 s, (Segment 3) a melting curve analysis generated by incubating at 95°C for 1 min followed by ramping the temperature down to 60°C and then back up to 95°C. The fluorescence data were collected at the end of each elongation step and collected continuously during the 60 to 95°C dissociation analysis. Data from each run were analyzed using MxPro-Mx3000P software version 3.00 (Stratagene-An Agilent Technologies Company, USA).

**5.2.6 Positive controls to generate the standard curves**

The standard curve for qPCR was constructed using the DNA of *L. intracellularis* pure culture strain PHE/MN1-00. Before the DNA extraction, the final concentration of *L. intracellularis* was estimated using a direct count method with immunostaining as described above. The DNA of $2x10^9$ *L. intracellularis* cells in pellet form was extracted using the DNeasy® kit. Purified DNA was collected by pooling the 2 x 200µl elution steps to maximize DNA yield. We assumed the kit provides a 100% DNA yield. The 400 µl of standard DNA was aliquoted, stored at -20°C, and thawed only once before use.

Based on the complete genome sequence data of *L. intracellularis* strain PHE/MN1-00 (http://www.ncbi.nlm.nih.gov/sites/entrez), *L. intracellularis* contains three plasmids and one chromosome. There is only one copy of the *L. intracellularis aspA* gene which is located on the chromosome. Therefore, it is reasonable to assume that a single copy of *aspA* represents one *L. intracellularis* organism. To generate the standard curve, *L. intracellularis* DNA was serially diluted 10-fold in ultra pure water to obtain standards representing the range of $5x10^8$ to $5x10^3$ *L. intracellularis*/ml. Then, 5 µl of each dilution were added to each reaction. The initial copy number of *L. intracellularis* was estimated to range between $2.5x10^6$ and $2.5x10^1$ *L. intracellularis*/well. Each dilution of the standard was run in triplicate in each run. The standard curve was generated using MxPro-Mx3000P software version 3.00. The software constructed a standard curve of threshold cycle (*Ct*) value versus initial concentration of the standard. *Ct* refers to the PCR cycle at which the fluorescence is across the defined threshold value and is inversely proportional to the initial DNA template concentration. Concentration values for the

unknown samples were extrapolated from the standard curve by the software and reported as the mean of 3 replicates.

### 5.2.7 Validation of the qPCR assay

To determine the limits of detection and quantification and the linear range of the qPCR assay, the results of the *L. intracellularis* standard curve (ranging from $2.5 \times 10^6$ to $2.5 \times 10^0$ *L. intracellularis*/well), pure culture (ranging from $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml) and spiked feces 1 (ranging from $2.55 \times 10^8$ to $2.55 \times 10^1$ *L. intracellularis*/g) were analyzed. The limit of detection of the assay was defined as the lowest concentration that returned at least 1 positive well on the qPCR assay. The limit of quantification of the assay, which is a measure of acceptable precision, was defined as the lowest concentration of *L. intracellularis* pure culture or spiked feces for which >50% of the qPCR reactions provided positive results with the correct *Tm*. The linear range of the assay, which is considered a working range in which the results are proportional to the concentration of the organism in the sample, was defined as the lowest concentration of pure culture or spiked feces that still showed a linear relationship with *Ct* and maintained a minimal standard deviation.

To determine the accuracy of the qPCR, the results of the *L. intracellularis* pure culture ranging from $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml as well as all of the spiked fecal samples were analyzed. The accuracy of the qPCR was assessed by first converting the *Ct* to *L. intracellularis*/well using the standard curve, then back-calculating with volumes and dilutions to determine *L. intracellularis*/ml for pure culture or *L. intracellularis*/g for the spiked fecal samples, and finally comparing the observed quantitative results to the expected number of *L. intracellularis* organisms.

To determine the effect of freezing and thawing of the fecal samples on qPCR accuracy, the results from all spiked feces were analyzed. Half of each spiked fecal sample was kept frozen at $-20^{o}C$ for approximately 2 weeks. These samples were then thawed, DNA was extracted, and qPCR was run on the newly extracted DNA. The results were compared to the corresponding DNA extractions from the fresh fecal samples.

To determine the inter-assay repeatability of qPCR, serial 10-fold dilutions of standard control DNA (ranging from $2.55 \times 10^{6}$ to $2.55 \times 10^{1}$ copy numbers of *L. intracellularis*/well) were analyzed in 12 independent, separate qPCR runs. Coefficients of variation (%CV) were calculated from the average *Ct* of each dilution across the 12 runs. The %CV of each dilution was expressed as the standard deviation / mean x 100. The intra-assay repeatability of qPCR was evaluated using DNA from 4 sources: pure culture at a concentration of $5.1 \times 10^{5}$ *L. intracellularis*/ml, spiked feces 1 at a concentration of $2.55 \times 10^{5}$ *L. intracellularis*/g, and spiked feces 3 and 5 at concentrations of $6.85 \times 10^{4}$ *L. intracellularis*/g. The DNA from each sample was independently extracted in triplicate. Each replicate DNA extraction was then assayed in 6 qPCR wells within a single run. The %CV of the 6 runs for each extraction was then analyzed.

### 5.2.8 Data analysis

All qPCR data were first analyzed using MxPro-Mx3000p software version 3.00 and then exported to perform final calculations with Microsoft® Excel® 2003 (Microsoft Corporation). In the dissociation curve analysis (a graph of melting temperature (*Tm*) versus fluorescence level), all samples that yielded a specific peak at $79^{o}C$ were included in a plot of *Ct* values against the initial logs of *L.*

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*intracellularis* concentrations. The linear range was measured, a linear regression was calculated, and the slope of the curve was generated. From this slope, the reaction efficiency of the qPCR was calculated using the equation: $E = (10^{-1/slope})-1$. Using the standard curve, the MxPro-Mx3000p software calculated the initial copy number of the *aspA* gene in each unknown sample for each PCR well. Then, the number of *L. intracellularis*/g of feces was calculated using the following equation (1): the number of *L. intracellularis*/g of feces = initial copy number of *L. intracellularis* per PCR well x A x B, where A = (total volume of DNA extracted/ 0.2 g of feces)/volume of DNA per PCR well and B = 7 (compensation factor for the volume of DNA lost during the DNA extraction using QIAamp$^{®}$).

The number of *L. intracellularis*/ml for the pure culture samples was calculated using the following equation (2): the number of *L. intracellularis*/ml = initial copy number of *L. intracellularis* per PCR well x A, where A = total volume of DNA/volume of DNA per PCR well.

To test whether there was a significant difference between the two fecal DNA extraction methods, the Wilcoxon signed-rank test was used. The qPCR results from each extraction method (expressed in *Ct* values) were compared, and a $P < 0.05$ was considered statistically significant. The coefficient of variations (%CV) was calculated to determine the repeatability of the qPCR intra- and inter-assay.

**5.3 Results**

**5.3.1 DNA extraction**

The difference between the two methods of extracting DNA from pure culture and feces was significant; *Ct* values were significantly lower following extraction with the DNeasy® and QIAamp® kits than with the modified Vanhoutte protocol for all concentrations of pure culture and spiked feces, respectively (P<0.01 in all concentrations). The DNeasy® kit extractions occasionally showed no *Ct* at concentrations of $5.1 \times 10^1$ *L. intracellularis*/ml, while modified Vanhoutte extractions occasionally had no *Ct* at concentrations of $5.1 \times 10^6$ *L. intracellularis*/ml. Whereas the QIAamp® kit extractions occasionally exhibited no *Ct* at concentrations as high as $2.55 \times 10^3$ *L. intracellularis*/g, the modified Vanhoutte extractions had no *Ct* at concentrations as high as $2.55 \times 10^4$ (Table 5.1). Because the DNeasy® and QIAamp® kits yielded more *L. intracellularis* DNA than that of the modified protocol of Vanhoutte et al. (2004), the DNeasy® and QIAamp® kits were selected for use in all subsequent DNA extractions from pure culture bacteria and pig fecal samples, respectively.

The DNA extraction efficiencies of the DNeasy® and QIAamp® kits were calculated based on the copy number of the *aspA* gene measured by qPCR compared to the expected copy number of the *aspA* gene with the assumption that the kits provide a 100% DNA yield. The DNA extraction efficiency of the DNeasy® kit with pure culture samples yielded an actual efficiency ranging from 33.35% to 88.71% with a mean extraction efficiency of 54.80±16.93% whereas the QIAamp® kit with

pig fecal samples had actual extraction efficiencies ranging from 8.2% to 19.23% with a mean extraction efficiency of 14.58±3.87%.

### 5.3.2 qPCR performance

**Specificity of the assay**-Conventional PCR and qPCR assays were performed to confirm the specificity of the selected primers. All 16 reference strain bacteria and 20 individual *L. intracellularis* negative control pig feces were negative with both conventional PCR and qPCR assays. Some pig feces showed *Ct* values after cycle 36 in the qPCR assay, but the melting curve analyses of these samples were all observed at an incorrect *Tm*.

**Standard curves**-DNA of *L. intracellularis* pure culture strain PHE/MN1-00 extracted with the DNeasy® kit was then used to evaluate the performance of the qPCR assay and to generate standard curves. Figure 5.1 shows amplification plots, a dissociation curve, and a standard curve of the serial 10-fold dilutions of standard DNA from $2.5x10^6$ to $2.5x10^1$ *L. intracellularis* copies/PCR well from 1 qPCR run. The qPCR assay yields a strong linear correlation ($R^2$ range 0.999 to 1) between *Ct* values and the initial copy number of *L. intracellularis* (Figure 5.1C). The assay gave occasional positive wells at the $2.55x10^0$ *L. intracellularis*/well concentration (not shown in Figure 5.1A), thus providing a limit of detection as low as several copies per well. The dissociation curve profile showed only a single melting temperature peak at 79°C (Figure 5.1B). There was no formation of primer-dimers, and non-template controls were uniformly negative in every qPCR run.

***L. intracellularis* pure culture and spiked fecal samples** –The amplification plots and dissociation curve analysis of pure culture and spiked feces 1 are shown in Figures 5.2 and 5.3, respectively. For pure culture samples, all of the dissociation curves from the DNA extracted from the bacteria concentrations of $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml showed the same single specific peak at $79^\circ$C (Figure 5.2B).

For spiked fecal samples, all 5 fecal samples also had the same specific melting temperature peak at $79^\circ$C, although fecal sample 1 spiked at low concentrations of *L. intracellularis* ($2.5 \times 10^4$ to $2.5 \times 10^1$ L. intracellularis/g) had a second peak at a higher and incorrect *Tm* (Figure 5.3B). This second peak, which likely indicates non-specific amplification, appeared only after cycle 36 in the qPCR assay.

### 5.3.3 Validation of the qPCR assay

In the pure culture samples, the limit of detection, limit of quantification, and linear range of the qPCR assay were evaluated with the serial 10-fold dilutions ranging from $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml. The detection limit was $5.1 \times 10^1$ *L. intracellularis*/ml, and with 83.8% of reactions positive, $5.1 \times 10^1$ *L. intracellularis*/ml was also the limit of quantification. A concentration of $5.1 \times 10^2$ *L. intracellularis*/ml was the lowest concentration that showed a linear range with low standard deviation of *Ct* values (Figure 5.4B).

For the spiked fecal samples, the limit of detection, limit of quantification, and linear range of the qPCR assay were evaluated with the serial 10-fold spikes ranging from $2.55 \times 10^8$ to $2.55 \times 10^1$ *L. intracellularis*/g of feces. The detection limit was $2.55 \times 10^1$ *L. intracellularis*/g but the limit of quantification was $2.55 \times 10^3$, with

88.8% of reactions positive at this concentration (Table 5.2). A concentration between $2.55 \times 10^4$ and $2.55 \times 10^3$ *L. intracellularis*/g was the lower limit of the linear range, demonstrating a low standard deviation of *Ct* values (Figure 5.5B).

To determine the ability of the qPCR assay to accurately quantify *L. intracellularis* in pure culture and pig fecal samples, the estimated copies of the *aspA* gene obtained from the qPCR assay were compared to the actual *L. intracellularis* concentrations in the samples. For the pure culture samples, the estimated counts were highly predictive of the actual count for all concentrations ranging from $5.1 \times 10^8$ to $5.1 \times 10^2$ *L. intracellularis*/ml and across replicates (Figure 5.4A). In general, the estimated counts were approximately 2-fold (with a range of 1.4 to 2.1-fold) lower than the expected across all dilutions.

In spiked fecal sample 1, the estimated counts were highly predictive of the actual count for all concentrations ranging from $2.55 \times 10^8$ to $2.55 \times 10^3$ *L. intracellularis*/g and among replicates (Figure 5.5A). The difference between the actual and expected qPCR values was < 2-fold (with a range 0.8- to 1.7-folds) for all concentrations above $2.5 \times 10^3$ *L. intracellularis*/g. For spiked fecal samples 2 through 5, the estimated counts were again highly predictive of the actual count for concentrations of > $6.85 \times 10^3$ *L. intracellularis*/g but were more variable for the concentration of $6.85 \times 10^2$ *L. intracellularis*/g (Figure 5.6). As with the spiked feces 1, the estimated counts were approximately ≤ 4-fold (with a range of 1.3- to 4.1-fold) lower than the expected for the fecal samples.

To determine the effect of a single freeze/thaw of the fecal sample on qPCR accuracy, DNA was extracted from the spiked fecal samples before and after freezing. The average *Ct* results of each replicate from each sample are shown in Table 5.2. After the fecal samples were frozen and thawed, the average *Ct* increased

by approximately 2 to 3 cycles in all concentrations above $10^3$ *L. intracellularis*/g. Many of the spiked samples with concentrations less than $10^3$ *L. intracellularis*/g appeared to be negative following the freeze/thaw cycle.

The inter-assay repeatability of the qPCR was evaluated by analyzing the *Ct* values of serial 10-fold dilutions of the standard control DNA containing $2.55 \times 10^6$ to $2.55 \times 10^1$ copy numbers of *L. intracellularis*/well from 12 independent, separate qPCR runs. Based on the analysis of standard curves from 12 different runs, a strong linear relationship ($R^2$ range 0.999 to 1, median 0.999; slope range -3.277 to -3.492, median -3.394; qPCR efficicency range 93.4 to 101.9, median 97.1) was observed on the regression analysis between *Ct* values and the initial copy number of *L. intracellularis* concentrations. The %CV of *Ct* values in each concentration ranged from 1.97 to 3.76%. The intra-assay repeatability of qPCR was evaluated by performing triplicate extractions on 4 different samples and then 6 qPCR reactions on each extraction with all reactions performed in the same qPCR run. The %CV of *Ct* values among the reactions from an extraction ranged from 1.03 to 1.60%, with minimal variation among extractions from a sample.

## 5.4 Discussion

Several conventional PCR assays have been developed, validated, and used to detect the presence of *L. intracellularis* DNA in a variety of samples (Jones et al., 1993ab; McOrist et al., 1994; Holyoake et al., 1996; Møller et al., 1998; Jacobson et al., 2004). Conventional PCR can only be used for semiquantitative measurements because of the limitations of PCR product endpoint analysis. To date, the direct count method with an immunostained assay is the primary method used to estimate the concentration of *L. intracellularis* in samples (Smith and McOrist, 1997; Guedes

et al., 2002d; Guedes and Gebhart, 2003ab; Chapter 2). The major disadvantages of the direct count method from fecal samples are that it is time-consuming, the enumeration results are subjective, and no specific antibody is available worldwide. A very recent study, a probe-based qPCR has been developed and validated for estimating the concentration of *L. intracellularis* in fecal samples. Although the assay shows a high specificity, it is still an expensive qPCR system and has limited in the range of their detection and quantification (Nathues et al., 2009). Therefore, there is a need for a quantitative assay that offers inexpensive, fast, simple and reliable detection and quantification of *L. intracellularis* in a variety of samples.

In this study, we developed a new SYBR green-based qPCR assay for the detection and quantification of *L. intracellularis* in pure culture and pig fecal samples. The assay showed high sensitivity, specificity, accuracy and repeatability. The assay had a limit of quantification of approximately $5.1x10^1$ *L. intracellularis*/ml and $2.55x10^3$ *L. intracellularis*/g for pure culture and fecal samples, respectively. The lower limit of the linear range was approximately $5.1x10^2$ *L. intracellularis*/ml and $2.55x10^3$ *L. intracellularis*/g for pure culture and fecal samples, respectively. The primer sets used in this study were designed to be specific for *L. intracellularis*, which was confirmed by the dissociation curve generating only a single peak at approximately 79$^o$C. No cross-reactivity of primers was observed when tested against other commensal organisms in the swine intestinal tract and bacteria closely related to *L. intracellularis*. However, some non-specific PCR products with a higher incorrect *Tm* for the *aspA* gene were found in the amplifications from 1 fecal sample (Figure 5.3B). When the PCR products were investigated further by running them in a 2% agarose gel, a higher molecular weight non-specific amplicon was observed at 300 bp, while the amplicon of the *aspA* gene

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was found to be 162 bp. In general, non-specific fluorescence signals can be found in qPCR with SYBR green due to the dye binding to any double-stranded DNA in the reaction. Although the non-specific signal of an incorrect product cannot always be avoided, it can be easily identified from the designed targets using the dissociation curve analysis.

The *aspA* gene was selected for this qPCR study because its sequence is highly specific to *L. intracellularis*. This gene is currently used as a target in a conventional PCR assay for the diagnosis of *L. intracellularis* infection at the University of Minnesota and is also used as an amplification target in a real-time PCR *L. intracellularis* detection assay (Pusterla et al., 2008). Only one copy of this gene was found on the chromosome of *L. intracellularis*; therefore, a single copy of the gene is representative of a single cell of *L. intracellularis*.

A comparison of the results obtained from qPCR with the expected numbers of *L. intracellularis* in pure culture and spiked fecal samples demonstrated the assay's high accuracy within its linear range. Copies detected, though, were roughly 2-fold less than expected; this could be due, in part, to assaying only the first DNA eluate. Because the QIAamp® kit only processes a fraction of the initial 0.2 g fecal samples (taking 200µl from 1.4 ml lysis buffer), an extraction efficiency of ~15% is to be expected. This fecal DNA extraction efficiency is higher than that reported in a recent *L. intracellularis* probe-based qPCR study (15% versus 3.5%) (Nathues et al., 2009). For DNA extractions from feces, the lower limit of this range was approximately $2.55 \times 10^3$ *L. intracellularis*/g. The inter- and intra-assay repeatability was high due to minimal variation among extractions, among qPCR runs and among replicates within a run. Consequently, the precision of this assay is also high. Below concentrations of approximately $10^3$ cells/g of feces, the qPCR could detect the

presence of *L. intracellularis* but could not quantify the concentration accurately or precisely. The reduced accuracy of qPCR for the quantification of low levels of bacteria (less than $10^3$ to $10^4$ organisms in a sample) has been reported in other qPCR studies (Wolffs et al., 2004; Harrington et al., 2005).

In this study, the detection limit of the SYBR green-based qPCR assay in spiked feces was $2.55 \times 10^1$ *L. intracellularis*/g of feces with a limit of quantification of $2.55 \times 10^3$ *L. intracellularis*/g. A previously published real-time PCR assay using a TaqMan probe system had a limit of detection of 1 *L. intracellularis* cell per PCR tube which equated to approximately $4 \times 10^4$ *L. intracellularis*/g of feces (Lindecrona et al., 2002). This difference in the detection limit of the assays may be due to the difference in DNA extraction methods. In the study by Lindecrona et al. (2002), feces were boiled in lysis buffer prior to DNA extraction. Although the assay is easier and cheaper than those used in this current study, it does not likely remove all potential PCR inhibitors from the feces which can lead to false-negative results. More importantly, the finding of at least 1 positive well at the $2.55 \times 10^1$ cells/g concentration could be due to cross-contamination. Probabilistically, it is unlikely that *L. intracellularis* DNA is present in the reaction well when the starting concentration is $2.55 \times 10^1$ cells/g. The detection of *L. intracellularis* DNA was more consistent across extractions (although still less than the limit of quantification) at the $2.55 \times 10^2$ cells/g concentration, and so perhaps $2.55 \times 10^2$ cells/g is a more accurate detection limit for this assay. In this study, the quantification limit of the SYBR green-based qPCR in fecal samples was $2.55 \times 10^3$ *L. intracellularis*/g whereas a recent probe-based qPCR had a limit of quantification at 10 genomic equivalents/µl reaction volume (approximately $2.8 \times 10^6$ genomic equivalents of *L.*

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*intracellularis*/g) (Nathues et al., 2009). This difference may be due to the fact that the assays target different genes or that they utilize different qPCR systems.

In this study, the commercially available QIAamp® and DNeasy® kits were compared with the modified protocol of Vanhoutte et al. (2004) for the extraction of *L. intracellularis* DNA from pig feces and pure culture samples, respectively. These assays were selected because commercial kits from QIAGEN have been widely used for DNA extraction from pig feces for the detection of pathogenic bacteria with conventional PCR. If the detection or quantification of *L. intracellularis* in those samples were required, no new extraction would need to be performed, which would save time and cost. Furthermore, since these commercial kits are available worldwide with the same quality, the qPCR results between laboratories can now be compared. Moreover, the kits have shown better results for fecal DNA extraction than some commercial kits from several other studies (McOrist et al., 2002; Li et al., 2003). Vanhoutte's metthod showed better results for total DNA yield and a higher DNA quality than QIAamp® when the assays were used for the extraction of total DNA from human feces (Vanhoutte et al., 2004). Unfortunately, the QIAamp® and DNeasy® kits give better overall qPCR results than the modified method of Vanhoutte et al. (2004) for the detection and quantification of *L. intracellularis* in pig feces and pure culture samples.

In Table 5.2, the *Ct* value of the $2.55 \times 10^1$ *L. intracellularis*/g samples was lower than the *Ct* value of the $2.55 \times 10^2$ *L. intracellularis*/g samples, mainly due to the fluorescence of a non-specific target. The dissociation curve in Figure 5.3B shows that there can be two *Tm* peaks, but this was only observed in the low concentration samples. This non-specific signal was stronger at lower bacterial concentrations. Because SYBR green binds all double-stranded DNA, the *Ct* values

are generated from the results of both peaks. Only 1 of the 5 fecal samples showed this non-specific amplification, but its occurrence in a fecal sample emphasizes the need for dissociation curve analysis at high *Ct* values and for careful interpretation when using those *Ct* values to calculate *L. intracellularis* concentrations.

A single freeze/thaw cycle of the fecal samples did impact the estimated concentration of *L. intracellularis* in the sample. The *Ct* value increased by approximately 2 to 3 cycles after one freeze/thaw cycle, indicating that approximately 75% of the *L. intracellularis* DNA in the sample was degraded. To obtain the best outcome, DNA should be extracted from fresh pig fecal samples. The results obtained from qPCR with frozen samples will likely underestimate the actual concentration of *L. intracellularis* in the samples. Another study, however, found that the amount of *L. intracellularis* DNA did not change when the fecal samples were frozen (Nathues et al., 2009). Future studies should continue to investigate the optimal way to store samples and the impact that freezing has on estimated *L. intracellularis* concentrations.

In conclusion, we have developed and validated a new SYBR green-based qPCR assay for the detection and quantification of *L. intracellularis* in pure culture and pig fecal samples. The results obtained from this assay are sensitive, specific, reliable, accurate, and precise for the detection and quantification of *L. intracellularis* in samples. This new qualitative and quantitative assay will be helpful in future disease transmission and epidemiology studies and should be applied to experimental and natural field condition samples to determine the dynamics and quantity of *L. intracellularis* excreted post-infection through feces.

## 5.5 Acknowledgements

**Figure 5.1** Amplification plots, dissociation curve, and standard curve of standard DNA with quantities of 2.5 $\times 10^6$ to 2.5$\times 10^1$ *L. intracellularis*/well. (A) Amplification plots (*Ct* cycle versus fluorescence level) of 10-fold dilutions of the standard DNA. (B) Dissociation curve analysis of the standard DNA demonstrating a single *Tm* peak at approximately 79$^\circ$C. (C) Standard curve was the plot of initial $\log_{10}$ quantity of *L. intracellularis* against threshold cycle (*Ct*). The standard curve is the result of triplicate analysis and the concentration of *L. intracellularis* in unknown samples was calculated based on this curve. The slope, correlation of efficient, and qPCR efficiency always ranges from -3.277 to -3.492, 0.999 to 1 and 93.4% to 101.9%, respectively.
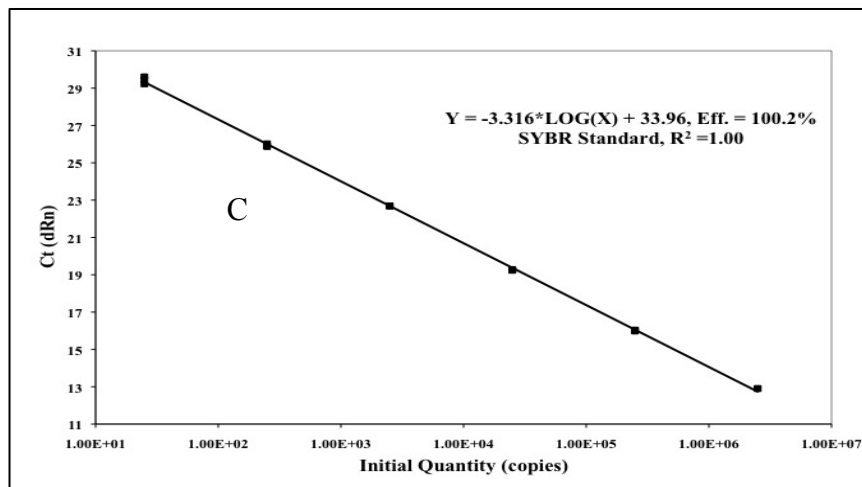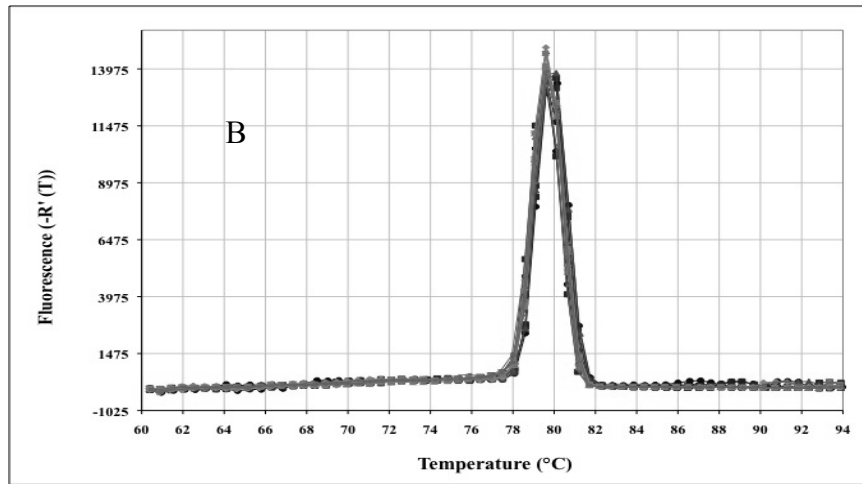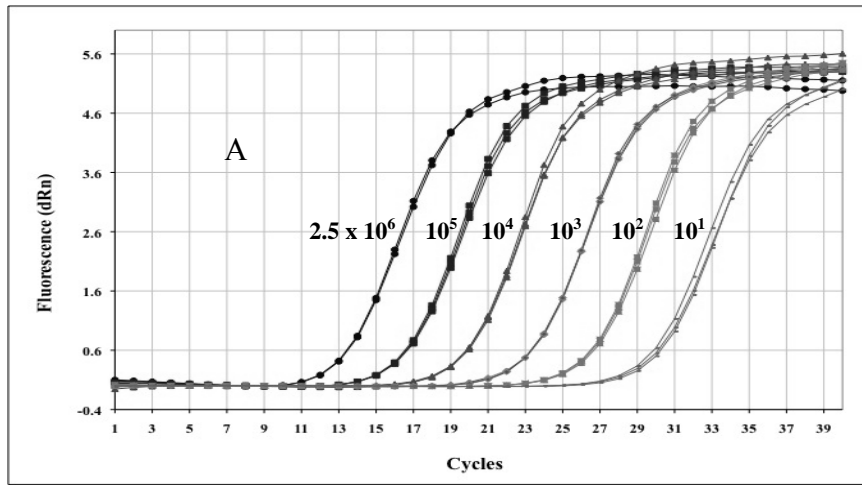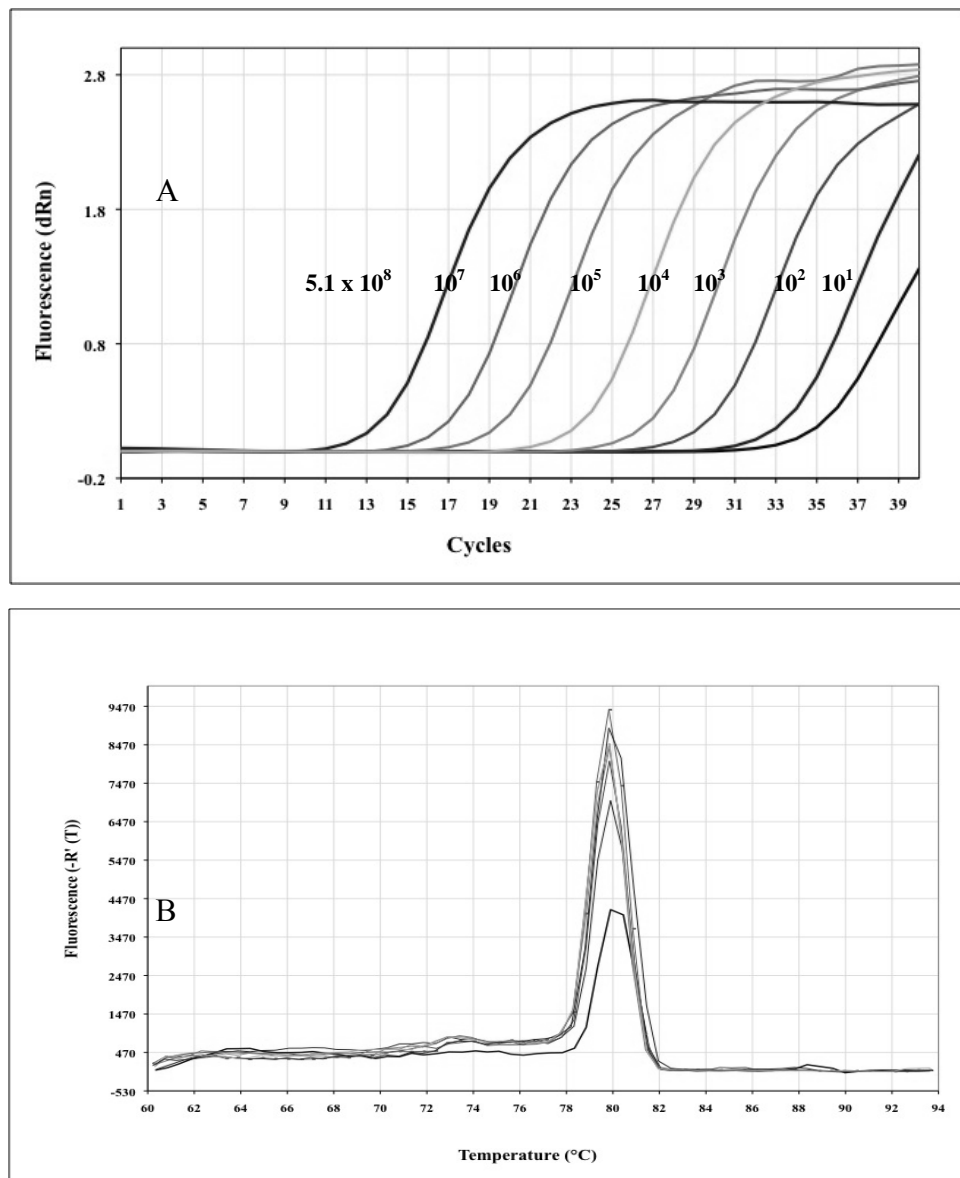
**Figure 5.2** Amplification plots and dissociation curves of DNA from *L. intracellularis* pure culture concentration of 5.1 x $10^8$ to 5.1 x $10^1$ *L. intracellularis*/ml extracted with the DNeasy® kit. (A) Amplification plots were generated from the average of triplicate qPCR wells. (B) Dissociation curve analysis of all pure culture concentrations showed a single specific peak of *Tm* at approximately 79°C, and no primer-dimers or non-specific peaks developed.
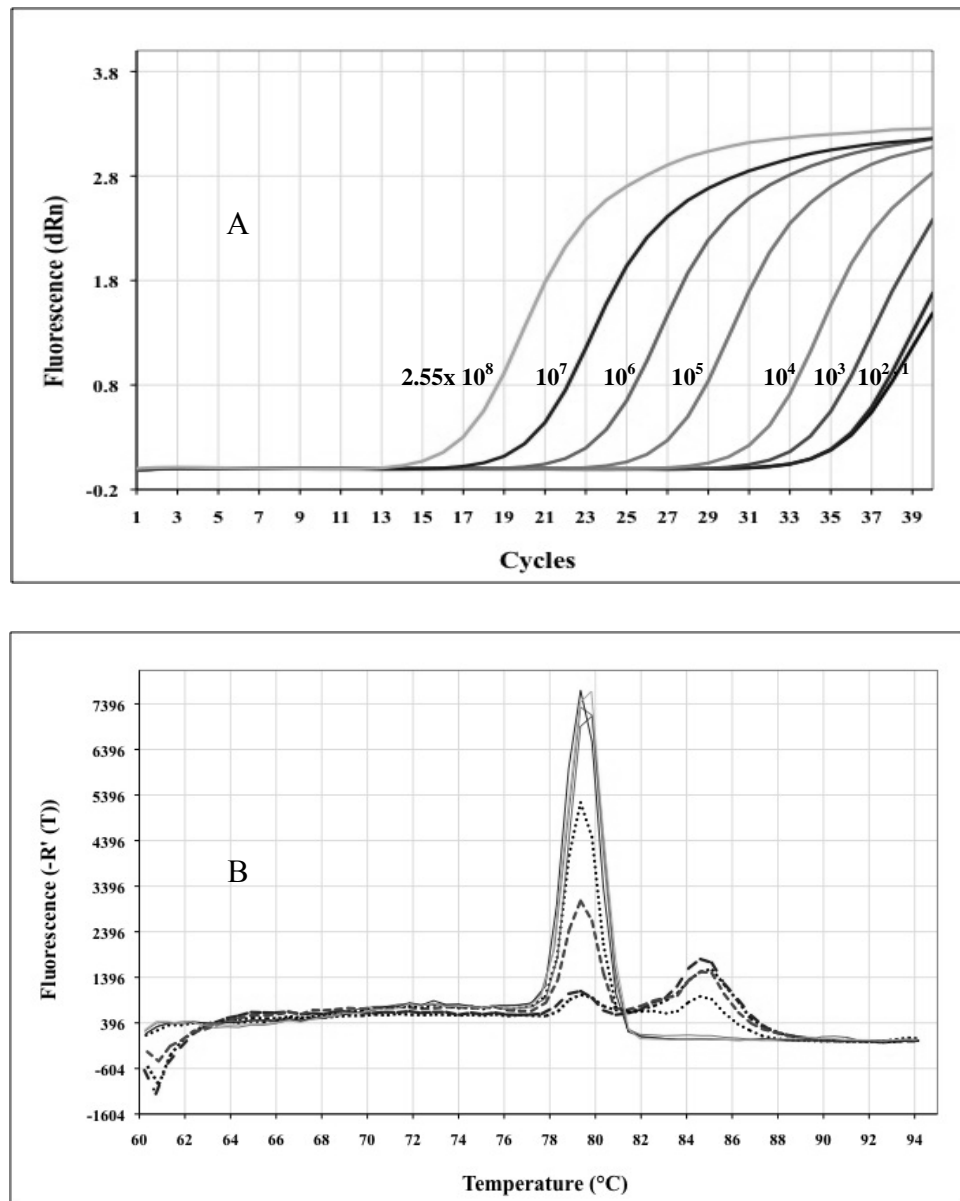
**Figure 5.3** Amplification plots and dissociation curves of DNA from spiked feces 1 contained 2.55 x $10^8$ to 2.55 x $10^1$ *L. intracellularis*/g, extracted with the QIAamp® kit. (A) Amplification plots were generated the average of triplicate qPCR wells. (B) Dissociation curve analysis of qPCR products from spiked feces 1 demonstrating a specific *Tm* peak at approximately 79°C and a non-specific peak with incorrect *Tm* (only found at low concentrations of 2.55x$10^4$ to 2.55x$10^1$ *L. intracellularis*/g of feces).

**Figure 5.4** Accuracy analysis and linear range of qPCR in pure culture samples. (A) Accuracy analysis of qPCR obtained from 2 replicate extractions of pure culture samples. The observed values obtained from the qPCR assay differed from the expected values by <2-fold across all concentrations of *L. intracellularis* pure culture. (B) Linear relationship between *Ct* values and pure culture concentrations. The qPCR assay began to lose the linear range of *Ct* values at a pure culture concentration of $5.1 \times 10^2$ *L. intracellularis*/ml. Each circle represents the *Ct* values from each qPCR well.
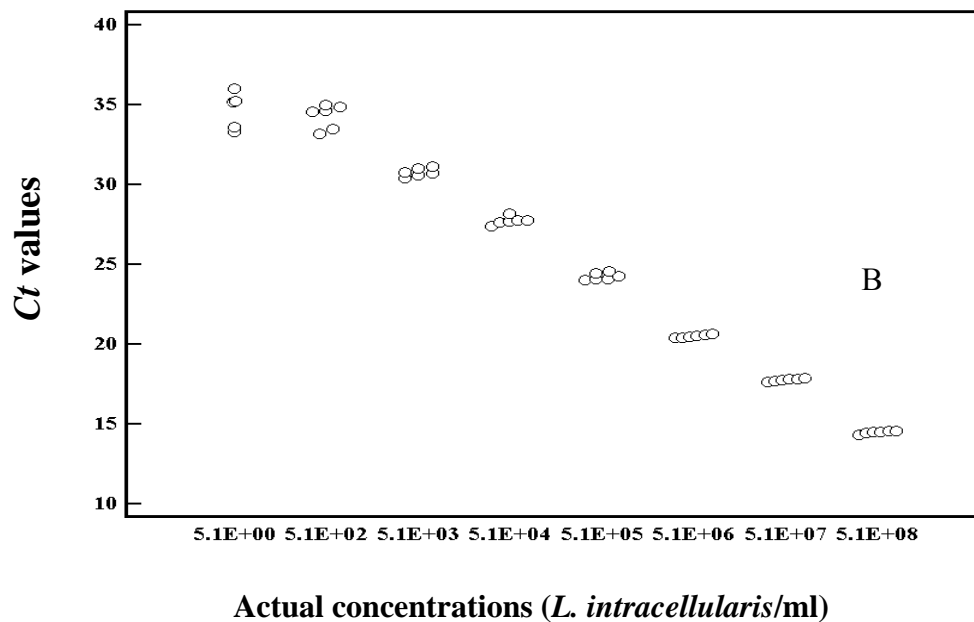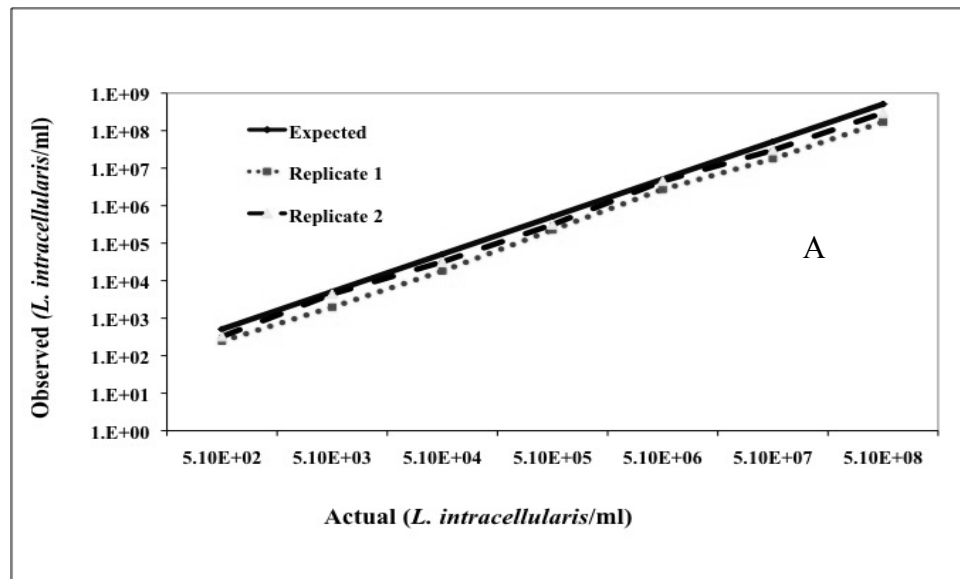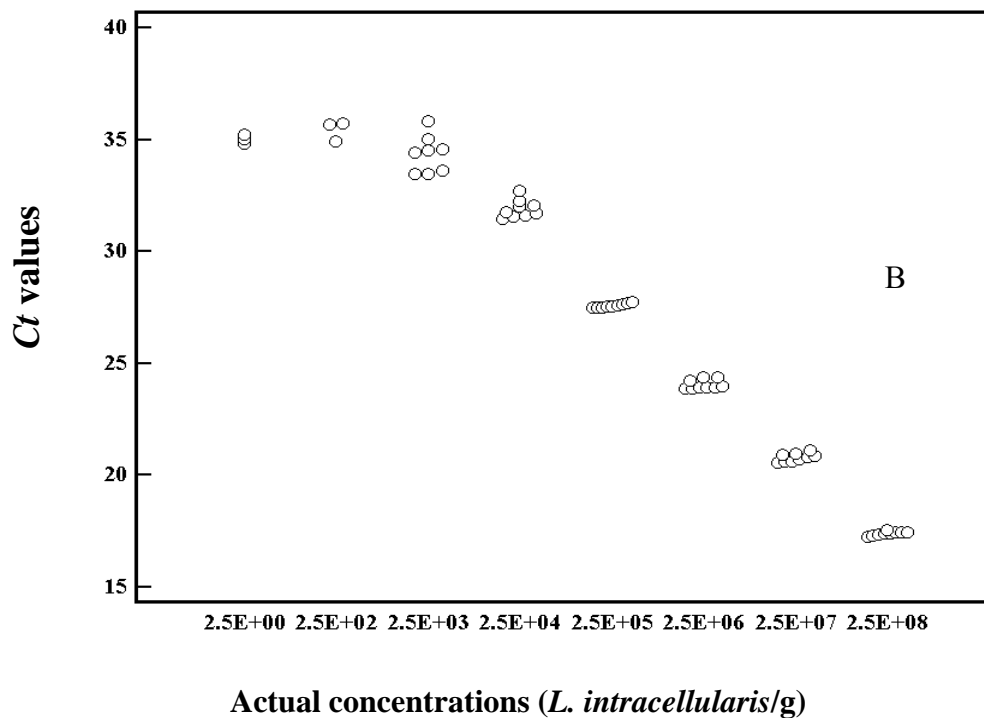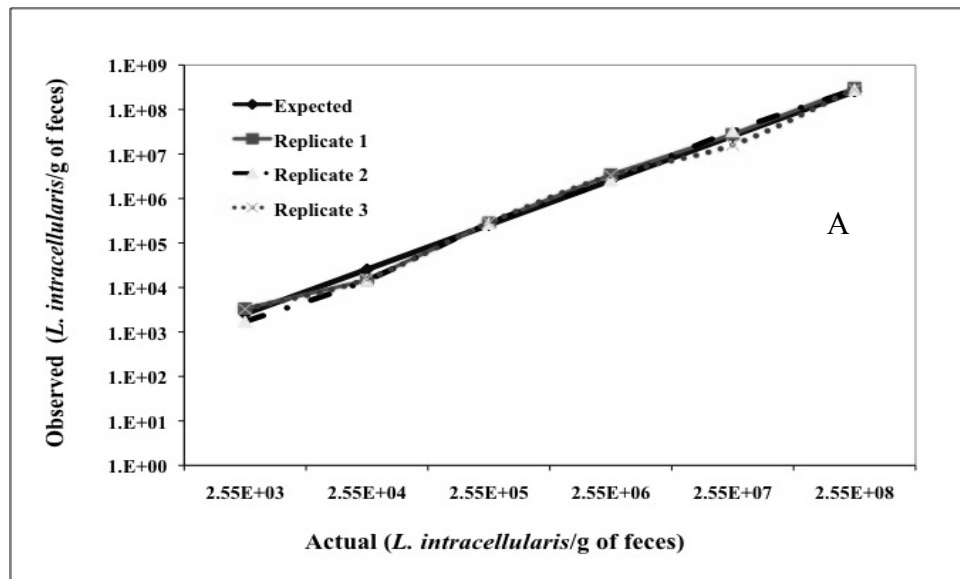
**Figure 5.5** Accuracy analysis and linear range of qPCR in spiked feces 1. (A) Accuracy analysis obtained from theee replicate extractions of spiked feces 1. The quantity of observed values obtained from the qPCR assay differed from the expected values by <2-fold across all concentrations of spiked feces 1. (B) Linear relationship between *Ct* values and bacteria in spiked feces. The qPCR assay began to lose the linear range of *Ct* values at concentrations between $2.55 \times 10^4$ and $2.55 \times 10^3$ *L. intracellularis*/g. Each circle represents the *Ct* values from each qPCR well.

**Figure 5.6** Accuracy analysis of qPCR in spiked feces 2 (A), 3 (B), 4 (C), and 5 (D) in which the feces contained a low concentration of *L. intracellularis,* from $6.85 \times 10^4$ to $6.85 \times 10^2$ *L. intracellularis*/g of feces. The initial quantity of *L. intracellularis* was calculated and converted to *L. intracellularis*/g of feces. The observed quantitative results differed by approximately 1- to 4-fold as compared to the expected number of *L. intracellularis*.
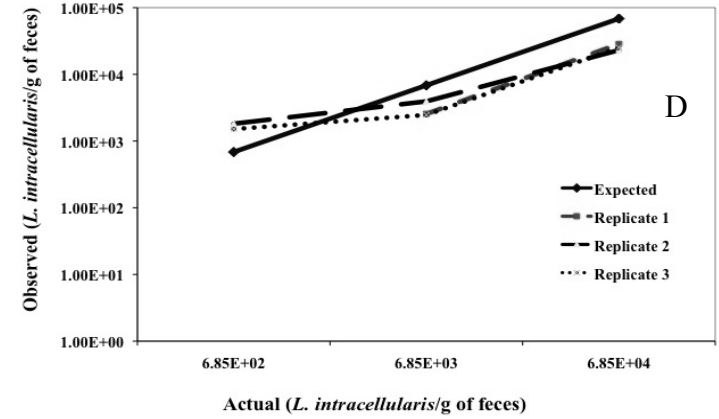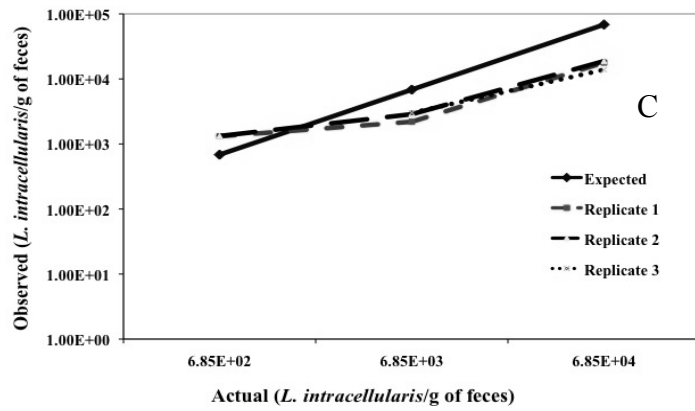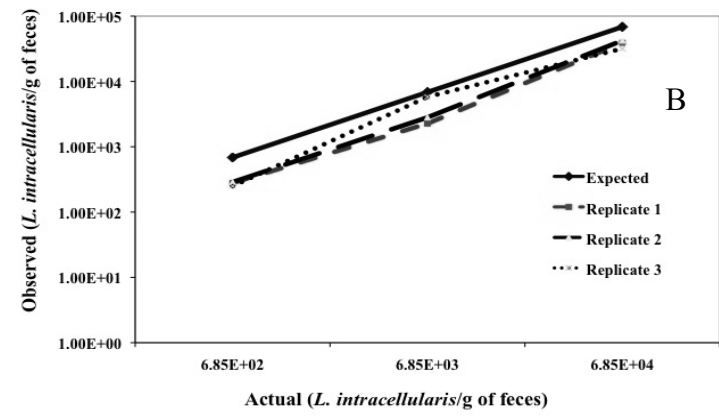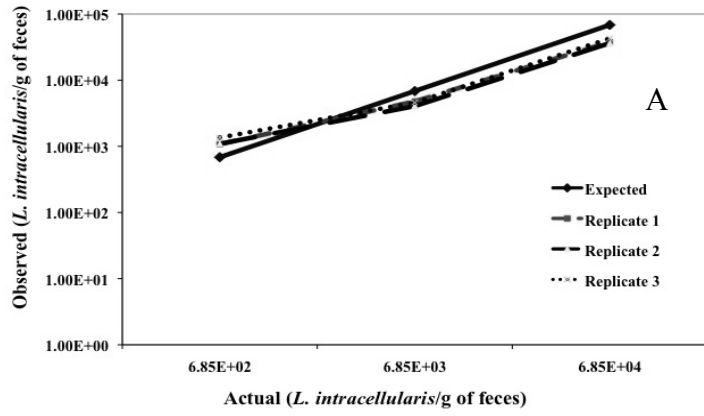
**Table 5.1** The average *Ct* values of spiked feces 1 and pure culture bacteria after using 2 different DNA extraction methods for the isolation of *L. intracellularis* DNA. The spiked feces 1 contained *L. intracellularis* concentrations ranging from $2.55 \times 10^8$ to $2.55 \times 10^1$ *L. intracellularis*/g of feces. Each feces was extracted with the QIAamp® kit and the modified protocol of Vanhoutte et al. (2004). The DNeasy® kit was compared to the modified protocol of Vanhoutte et al. (2004) for the isolation of DNA from cultured bacteria in which the concentrations ranged from $5.1 \times 10^8$ to $5.1 \times 10^1$ *L. intracellularis*/ml. The average *Ct*±SD was obtained from 3 replicates of DNA extraction from each concentration, and each replicate was run in triplicate reaction.

| Fecal DNA extraction | | | Pure culture DNA extraction | | |
|---|---|---|---|---|---|
| *L. intracellularis* concentration | QIAamp® | Vanhoutte et al. (2004) | *L. intracellularis* concentration | DNeasy® | Vanhoutte et al. (2004) |
| | *Ct* average ± SD | *Ct* average ± SD | | *Ct* average ± SD | *Ct* average ± SD |
| $2.55 \times 10^8$ | 18.98±0.89 | 22.76±0.54 | $5.1 \times 10^8$ | 14.50±0.09 | 25.13±1.97 |
| $2.55 \times 10^7$ | 22.70±0.47 | 27.12±0.72 | $5.1 \times 10^7$ | 17.19±0.08 | 33.07±1.88 |
| $2.55 \times 10^6$ | 25.89±0.67 | 28.86±1.59 | $5.1 \times 10^6$ | 20.53±0.08 | 37.31±0.92 |
| $2.55 \times 10^5$ | 29.39±0.44 | 33.42±1.26 | $5.1 \times 10^5$ | 24.29±0.21 | No *Ct* |
| $2.55 \times 10^4$ | 32.41±0.64 | 35.35±0.76 | $5.1 \times 10^4$ | 27.68±0.13 | No *Ct* |
| $2.55 \times 10^3$ | 34.22±0.67 | 35.02± | $5.1 \times 10^3$ | 30.71±0.20 | No *Ct* |
| $2.55 \times 10^2$ | 34.73±0.75 | 36.13±1.45 | $5.1 \times 10^2$ | 34.25±0.75 | No *Ct* |
| $2.55 \times 10^1$ | 34.50±0.70 | No *Ct* | $5.1 \times 10^1$ | 34.69±1.04 | No *Ct* |

**Table 5.2** The average *Ct* values of spiked fecal samples 1 to 5 before and after freezing. The DNA of *L. intracellularis* was extracted from each sample before and after freezing to determine the effects of freezing/thawing on the estimated *L. intracellularis* in fecal samples. Three independent extractions were performed for each concentraion of samples and each replicate was tested in triplicate qPCR reactions.

| Fecal Samples *L. intracellularis* concentrations | Before freezing the feces *Ct* values ±SD (# positive/#qPCR reactions) | | | After freezing the feces *Ct* values±SD (# positive/#qPCR reactions) | | |
|---|---|---|---|---|---|---|
| Spiked feces 1 | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 1 | Replicate 2 | Replicate 3 |
| $2.55 \times 10^8$ | 17.32±0.09 (3/3) | 17.45±0.12 (3/3) | 17.44±0.01 (3/3) | 19.41±0.03 (3/3) | 19.53±0.08 (3/3) | 19.53±0.09 (3/3) |
| $2.55 \times 10^7$ | 20.83±0.09 (3/3) | 20.61±0.02 (3/3) | 21.00±0.17 (3/3) | 22.81±0.07 (3/3) | 22.4±0.09 (3/3) | 22.82±0.18 (3/3) |
| $2.55 \times 10^6$ | 23.91±0.02 (3/3) | 24.33±0.10 (3/3) | 23.97±0.02 (3/3) | 26.06±0.03 (3/3) | 26.22±0.09 (3/3) | 26.06±0.03 (3/3) |
| $2.55 \times 10^5$ | 27.56±0.03 (3/3) | 27.62±0.10 (3/3) | 27.56±0.14 (3/3) | 29.84±0.04 (3/3) | 29.63±0.16 (3/3) | 29.79±0.11 (3/3) |
| $2.55 \times 10^4$ | 31.98±0.66 (3/3) | 31.93±0.29 (3/3) | 31.85±0.34 (3/3) | 34.02±1.59 (3/3) | 34.66±1.63 (3/3) | 33.95±0.46 (3/3) |
| $2.55 \times 10^3$ | 34.14±0.56 (3/3) | 35.14±0.63 (3/3) | 33.55±0.12 (2/3) | No *Ct* (0/3) | 35.22±1.21 (2/3) | 36.3±0.81 (2/3) |
| $2.55 \times 10^2$ | 35.75 (1/3) | 35.28±0.53 (2/3) | No *Ct* (0/3) | No *Ct* (0/3) | No *Ct* (0/3) | No *Ct* (0/3) |
| $2.55 \times 10^1$ | 35.01±0.26 (2/3) | 35.03 (1/3) | No *Ct* (0/3) | No *Ct* (0/3) | No *Ct* (0/3) | No *Ct* (0/3) |
| Spiked feces 2 | | | | | | |
| $6.85 \times 10^4$ | 29.99±0.42 (3/3) | 30.01±0.13 (3/3) | 29.76±0.21 (3/3) | 32.86±0.97 (3/3) | 32.43±0.79 (3/3) | 32.7±0.32 (3/3) |
| $6.85 \times 10^3$ | 33.17±0.69 (3/3) | 33.46±0.88 (3/3) | 33.23±0.66 (3/3) | 34.01 (1/3) | 35.88±0.01 (2/3) | 34.66±0.92 (2/3) |
| $6.85 \times 10^2$ | 35.06±1.62 (2/3) | 35.93±2.05 (3/3) | 34.45±0.86 (2/3) | No *Ct* (0/3) | No *Ct* (0/3) | 35.13±0.29 (2/3) |

**Table 5.2** Continued.

| Fecal Samples *L. intracellularis* concentrations | Before freezing the feces $Ct$ values ±SD (# positive/#qPCR reactions) | | | After freezing the feces $Ct$ values±SD (# positive/#qPCR reactions) | | |
|---|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 1 | Replicate 2 | Replicate 3 |
| Spiked feces 3 | | | | | | |
| $6.85 \times 10^4$ | 29.91±0.24 (3/3) | 29.77±0.13 (3/3) | 30.22±0.31 (3/3) | 33.87±0.98 (3/3) | 32.73±0.18 (3/3) | 33.43±1.38 (3/3) |
| $6.85 \times 10^3$ | 34.17±2.24 (2/3) | 34.01±0.82 (3/3) | 32.85±0.67 (3/3) | 35.85± (1/3) | No $Ct$ (0/3) | 35.96±0.1 (2/3) |
| $6.85 \times 10^2$ | 35.97± (1/3) | 35.84± (1/3) | 37.51±2.02 (2/3) | No $Ct$ (0/3) | No $Ct$ (0/3) | No $Ct$ (0/3) |
| Spiked feces 4 | | | | | | |
| $6.85 \times 10^4$ | 30.87±0.53 (3/3) | 30.74±0.09 (3/3) | 31.24±0.49 (3/3) | 33.48±0.33 (3/3) | 33.12±0.52 (3/3) | 33.81±1.22 (2/3) |
| $6.85 \times 10^3$ | 34.02±0.65 (3/3) | 33.59±0.57 (3/3) | 33.54±0.68 (3/3) | 35.72±0.2 (2/3) | No $Ct$ (0/3) | 34.75 (1/3) |
| $6.85 \times 10^2$ | 34.7 (1/3) | 34.69 (1/3) | No $Ct$ (0/3) | 34.79 (1/3) | No $Ct$ (0/3) | No $Ct$ (0/3) |
| Spiked feces 5 | | | | | | |
| $6.85 \times 10^4$ | 30.13±0.31 (3/3) | 30.46±0.34 (3/3) | 30.34±0.43 (3/3) | 35.67±0.05 (2/3) | 34.86±1.45 (2/3) | 34.56±1.52 (3/3) |
| $6.85 \times 10^3$ | 33.81±0.73 (3/3) | 33.14±0.60 (3/3) | 33.82±0.54 (3/3) | 37.92 (1/3) | No $Ct$ (0/3) | No $Ct$ (0/3) |
| $6.85 \times 10^2$ | No $Ct$ (0/3) | 34.21 (1/3) | 34.49 (1/3) | No $Ct$ (0/3) | No $Ct$ (0/3) | No $Ct$ (0/3) |

SECTION IV

SUMMARY AND CONCLUSIONS

**Summary and conclusions**

Several studies throughout this Ph.D. thesis were initiated because of the need for more information about the *in vitro* activities of antimicrobials and disinfectants against *L. intracellularis*. This work is an important data source for selecting the most active antimicrobials and disinfectants to treat and control porcine PE. Moreover, there is a need for a new quantitative assay to accurately and precisely quantifies *L. intracellularis* in a variety of samples. The results of these thesis projects have expanded upon the limited information available about antimicrobials and disinfectants and fulfilled the need for a quantitative assay.

Section I, chapter 1 summarizes previous studies and a basic knowledge of *L. intracellularis*. The literature review addresses the pathogenesis, epidemiology, diagnosis, treatment, and control of PE in pigs.

The aims of the investigations presented in Section II, chapters 2 through 4, were to obtain more information about the *in vitro* minimum inhibitory concentration (MIC) of antimicrobials and *in vitro* bactericidal activity of disinfectants against *L. intracellularis*. In chapter 2, we developed a modified tissue culture assay in 96-well tissue culture plates for determining the MICs of 6 antimicrobials against 10 isolates of *L. intracellularis* derived from pigs in the U.S. and Europe. The MIC of each antimicrobial was defined as the lowest antimicrobial concentration that inhibited 99% of bacterial growth, as compared to the antimicrobial-free control. Individual isolates of *L. intracellularis* have diverse antimicrobial sensitivity response patterns. Therefore, antimicrobial selection is important in order to obtain the best possible treatment outcome. Both intracellular and extracellular antimicrobial activities were determined in order to mimic

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infection situations in which the bacteria are exposed to the antimicrobial compound either before or after infection of intestinal cells. According to our findings, we predict that carbadox, tiamulin, and valnemulin are the most active antimicrobials, chlortetracycline and tylosin are intermediately active antimicrobials, and lincomycin is the least active antimicrobial against *L. intracellularis in vitro.* The MIC results of 2 separate bacterial replicates from the modified tissue culture assay were consistently within a 2–fold dilution range, suggesting that this is a reliable and reproducible assay for determining the MICs of antimicrobials against *L. intracellularis.* A limitation of this study was that 10 *L. intracellularis* isolates might not provide a sufficient sampling size to generalize antimicrobial sensitivity results. However, we believe that the results of this study can be used as an *in vitro* guideline for antimicrobial selection decisions in the treatment and control of PE in pigs.

Chapter 3 describes the use of the modified tissue culture assay and the direct count method with special fluorescence dye (Live/Dead[®] BacLight™) to evaluate the bactericidal activity of powder disinfectant (Stalosan[®] F) against 2 isolates of *L. intracellularis.* Although Stalosan[®] F is recommended for use as a dry powder applied at 50 g/m$^2$, a suspended form of Stalosan[®] F was also tested to simulate the potential field scenario of the powder mixing with the pig's drinking water or urine. The results of the assays indicate that both the powder and suspension forms were able to inactivate *L. intracellularis.* Both strains of *L. intracellularis* were more than 99% inactivated after 30 min of exposure to both ≥ 0.25g/cm$^2$ of powder and ≥16% of aqueous suspension. While the mode of action of this disinfectant remains unclear, scanning electron micrographs show that the

disinfectant might have the potential to lyse the *L. intracellularis* cell membrane. The results obtained from both the modified tissue culture assay and the direct count method were similar and showed a high degree of correspondence. This suggests that either assay would be appropriate to determine the bactericidal activity of disinfectants against *L. intracellularis.*

Chapter 4 sought to evaluate the *in vitro* bactericidal activity of 7 commonly used disinfectants against 2 isolates of *L. intracellularis* using a modified tissue culture method. The disinfectants represented several classes, including iodine, quaternary ammonium compounds, aldehyde, phenol, oxidizing agents, and biguanides. *In vitro* conditions were designed to simulate field conditions with 2 levels of water hardness (400 ppm and 1000 ppm of $CaCO_3$) and the presence of an organic load (5% FBS). The disinfectants were tested for 10 min at the labeled dose in synthetic water conditions. The results indicated that both isolates of *L. intracellularis* were highly susceptible of DC&R®, Roccal®-D, Synergize®, and Virkon®-S, while the bacteria were more tolerant to Certi-Dine®, Nolvasan® S, and Tek-Trol®. When the simulated water hardness was increased from 400 ppm to 1000 ppm, the efficacies of most of the disinfectants decreased slightly, whereas only Synergize® remained constant. The bactericidal activities of all disinfectants increased with increasing disinfectant concentrations and exposure times. Based on our results, we would predict that DC&R®, Roccal®-D, Synergize®, and Virkon®-S would perform well under field conditions for inactivating viable *L. intracellularis*. This information can serve as an *in vitro* guide for disinfectant selection in controlling free-living *L. intracellularis* in the environment.

The elimination and inactivation of *L. intracellularis* both inside and outside of animals is important for the treatment and control of PE. Antimicrobials to which *L. intracellularis* is susceptible only eliminate the organism from inside animals, while disinfectants only inactivate free-living *L. intracellularis* in the environment. The use of antimicrobials and disinfectants should be conducted simultaneously. The results from our investigations provide antimicrobials and disinfectants of choice for selection decisions. Although it is clear which antimicrobials and disinfectants are the most active against *L. intracellularis in vitro*, successful treatment and control strategies against PE in pigs is dependent not only on the individual compounds but also on their interaction with several other factors. For instance, the effectiveness of an antimicrobial is also dependent on the dose, route of administration, timing, and pharmacokinetic and pharmacodynamic activities of the antimicrobials. The bactericidal activities of disinfectants depend on temperature, levels of organic materials in the water and environment, hardness of water, type of floor, and application concentration. Therefore, it is necessary to understand the nature of the disease and the limitations of antimicrobials and disinfectants in order to create the most effective strategy for treating and controlling the disease.

Section III, Chapter 5 describes the development and validation of a new quantitative assay, qPCR using SYBR green, to accurately and precisely quantify *L. intracellularis* in a variety of sample types. The complete genome of *L. intracellularis* strain PHE/MN1-00 indicates that the bacteria contain three plasmids and one chromosome with only one copy of the *aspA* gene found on the chromosome. Specific primers were designed using the *aspA* gene as the target, and it was assumed that one copy of the *aspA* gene represents one *L. intracellularis*

organism. The selected primers had very high specificity. All 16 species of enteric bacteria that are commonly found in pigs or that are closely related to *L. intracellularis* and all 20 negative control fecal samples were negative using the qPCR assay. The assay was validated with serial 10-fold dilutions of pure culture samples and several sets of spiked feces containing between $10^8$ and $10^1$ *L. intracellularis*/ml and *L. intracellularis*/g of feces, respectively. The limits of detection and quantification of *L. intracellularis* pure culture were $5.1 \times 10^1$ *L. intracellularis*/ml and $5.1 \times 10^2$ *L. intracellularis*/ml, respectively. In spiked feces, the assay could detect bacteria levels as low as $2.55 \times 10^1$ *L. intracellularis*/g of feces, while the limit of quantification was $2.55 \times 10^3$ *L. intracellularis*/g of feces. The estimated counts of *L. intracellularis* using the qPCR assay were highly predictive of the actual pure culture concentrations for all concentrations over $5. \times 10^2$ *L. intracellularis*/ml and the actual fecal concentrations for all concentrations above $2.55 \times 10^3$ *L. intracellularis*/g in spiked feces. The accuracy of the qPCR assay was approximately 2-fold lower than the expected values across all dilutions of pure culture and spiked feces. The assay shows high precision since it shows high inter- and intra- repeatability with minimal variation among DNA extractions, among qPCR runs, and among replicates within a run. The dissociation curve analysis shows that no primer-dimers were found and only a single peak of *Tm* was consistently found at 79°C when tested with both pure culture and spiked feces. However, two *Tm* peaks from non-specific signals were sometimes found at low concentrations of *L. intracellularis* in spiked feces, though these were easily identified from the designated target under dissociation curves. Fresh fecal samples were more effective for DNA extraction since approximately 75% of *L.*

*intracellularis* DNA was degraded after freezing one time. Our validation results indicate that this new qPCR assay is sensitive, specific, reliable, accurate, precise, and inexpensive for the detection and quantification of *L. intracellularis* in different types of samples.

In summary, these investigations have expanded upon the limited information available about *in vitro* antimicrobial sensitivities and *in vitro* bactericidal activities of disinfectants. Moreover, we have developed and improved an *in vitro* assay for measuring antimicrobial and disinfectant activity against *L. intracellularis.* We hope that our results will help veterinarians to more effectively make decisions regarding antimicrobial and disinfectant use in the treatment and control of PE in pigs. The new quantitative PCR for the detection and quantification of *L. intracellularis* in samples will enhance future studies of the epidemiology and disease transmission of PE in pigs.

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