The development of a semi-quantitative, real-time diagnostic assay for ileitis
2C-109

Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

By

Dr Alison Collins
NSW Department of Primary Industries
Elizabeth Macarthur Agricultural Institute,
PMB 4008, Narellan NSW 2567
alison.collins@industry.nsw.gov.au
02 46 406 306

August 2011

Established and supported under the Australian Government's Cooperative Research Centres Program

Executive Summary

Proliferative enteropathy (PE) or ileitis is a common disease of grower and finisher pigs that causes diarrhoea, reduced ADG and FCE, increased P2 backfat and increased variation and days to slaughter. Ileitis can be controlled with a live oral vaccine (Enterisol® Ileitis, Boehringer Ingelheim), with antibiotic medication or with improved hygiene in piggeries. Ileitis can cost producers between $8 and $13 per pig if sub-clinical and clinical ileitis are not controlled. This project aimed to develop two new diagnostic tests that could help in the management of ileitis. The first test is a rapid, easy to use 'on-farm' lateral flow immunoassay that can confirm whether ileitis is the cause of scouring in individual pigs. The second test is a laboratory test that can quantify the number of *L.intracellularis* bacteria (that cause ileitis) shed in pig faeces.

The lateral flow immunoassay (LFIA) we developed captures *L.intracellularis* from faecal samples on blue beads coated with an antibody specific to *L.intracellularis*. The antibody-antigen-bead complex is captured by *L.intracellularis* antibodies applied directly to a hydrophobic membrane (test line). Faecal samples without *L.intracellularis* are captured by a goat anti-rabbit antibody applied directly to the membrane 5mm away from the test line. This second antibody provides an internal control for every faecal sample to demonstrate that the test has worked. Preliminary steps in the development of the LFIA included evaluating a range of inexpensive reagents and procedures to partially purify faecal samples; producing and purifying polyclonal antibodies to *L. intracellularis* in rabbits and evaluating their sensitivity and specificity; adsorbing purified *L.intracellularis* antibodies to blue beads (microspheres); and testing the ability of these antibody coated beads to capture *L. intracellularis* from faeces.

A simple and inexpensive faecal purification method was developed that can be performed easily on farm. *L. intracellularis* was detected consistently in faeces from pigs with clinical and sub-clinical ileitis following dilution and filtration through a glass wool and sand column.

Polyclonal antibodies were produced in rabbits by repeated injection with purified *L.intracellularis* extracted from pigs affected with ileitis. The rabbit anti-*L.intracellularis* serum (R97) produced is as sensitive as the commercially available monoclonal antibodies to *L.intracellularis* (IG4 and VPM53), and reacts predominantly with the same 22kDa *L.intracellularis* protein as the two monoclonal antibodies. The rabbit anti-*L.intracellularis* serum (R97) does not cross-react with other common pig enteric pig pathogens. However, it appears the R97 sera does cross-react with other proteins found in the faeces of pigs.

Beads were coated with the rabbit anti-Lawsonia antibodies to saturation and lateral flow membrane strips were constructed with optimised concentrations of beads and antibodies. However we had difficulty overcoming false positive results, where *L.intracellularis* negative faecal samples reacted with the antibody coated beads, producing a positive test line. It is possible that the immunised rabbit produced antibodies to an additional protein in the *L.intracellularis* inoculum that cross-reacts with proteins in the pig faeces. A number of methods were tried to improve the specificity of the LFIA, including removal of non-IgG proteins from the rabbit anti Lawsonia sera; blocking any vacant binding sites on antibody coated beads with bovine serum albumin; blocking the nitrocellulose membrane and the sample buffer with a wide range of detergents, salts and proteins; absorbing the rabbit anti Lawsonia sera with healthy pig gut cells; and purifying the sera on an affinity column bound with *L.intracellularis*. To date, none of these have been successful in producing a LFIA which is specific and sensitive enough for our needs. It is possible that a monoclonal antibody to *L.intracellularis* (produced in mice) would not react with negative pig faeces, and this would be worth investigating with a commercial partner. We have also investigated a wide range of detergents and inert proteins to prevent non-specific antigen-antibody binding both on the beads and the nitrocellulose membranes.

In the second part of the project, a high throughput laboratory assay was developed to quantify the number of *L.intracellularis* shed in pig faeces. This quantitative PCR (qPCR) is 97% specific and 99% sensitive compared to the conventional PCR currently in use, and does not cross-react with a wide range of pig gut pathogens including *E.coli, Salmonella spp.*, *Campylobacter spp., Brachyspira hyodysenteriae* and *B.pilosicoli*. The qPCR is able to reliably detect as few as 2,600 *L.intracellularis* per 0.2g of faeces (sub-clinical infection), and can detect a single ileitis affected pig in a pool of 10 pigs. The qPCR is also able to detect 4 sub-clinically affected pigs in a pool of 10

faecal samples. We also developed an automated DNA extraction method that is capable of extracting DNA from 96 samples simultaneously. This is the same format for the automated qPCR, which significantly reduces the assay time (4 fold), compared with the conventional PCR.

When evaluated with scouring and healthy pigs from commercial herds, the qPCR was able to demonstrate significantly higher numbers of *L.intracellularis* in the faeces of scouring pigs relative to non-scouring pigs. In pigs experimentally infected with *L.intracellularis*, the qPCR correlated well with other measures of ileitis severity including scouring and gut pathology, and negatively with ADG (as expected). We also used the qPCR to show the relationship between average daily gain and the numbers of *L.intracellularis* shed in experimentally infected pigs. Future research focussed on identifying a critical threshold of *L.intracellularis* that leads to production losses on commercial farms would be an invaluable resource for pig producers. Faecal samples and production data would need to be collected from a wide range of herds (with varying genetics, environment and diet) to identify the critical threshold of *L.intracellularis* that causes production losses.

We have also used the qPCR to show the benefits of vaccination and medication in small groups of pigs experimentally infected with *L.intracellularis*. Both control measures reduced the number of *L.intracellularis* in faeces, which improved the health and growth of pigs relative to untreated animals. However, further work is needed on commercial herds to demonstrate the value of the qPCR to evaluate ileitis control measures.

The potential risk that rats pose to pigs as a source of *L.intracellularis* was also demonstrated with the qPCR. This study showed for the first time that rats can shed enough *L.intracellularis* in one gram of faeces to infect pigs, which demonstrates the importance of rodent extermination on farm.

Although more work needs to be done on commercial herds, the qPCR will help producers decide the most cost-effective treatment for pigs with ileitis and will quantify the efficacy of vaccination, antibiotic medication and improved hygiene to control ileitis and reduce the associated production losses.

Table of Contents

1. Introduction

Proliferative enteropathy (PE), also known as ileitis, affects pigs of all ages across a broad range of management systems. Growing pigs suffer from reduced weight gains, diarrhoea, poor FCE, increased days to slaughter and increased P2 backfat, which can result in net revenue losses of $13 per pig in clinically affected herds (Holyoake et al., 2010a). Pigs can also be sub-clinically affected with ileitis, with reduced and variable growth, reduced feed intake and reduced muscle growth in the absence of diarrhoea (Collins et al., 2010), which results in revenue losses of $8 per pig in herds sub-clinically affected with ileitis (Holyoake et al., 2010a). A recent survey found that 100% of Australian herds are infected with *Lawsonia intracellularis*, the bacteria that causes ileitis, with an average of 84% of animals infected per herd (Holyoake et al., 2010b). Control of ileitis is either by medication with antibiotics, or vaccination with an avirulent live *L. intracellularis* vaccine (Enterisol® Ileitis, Boehringer Ingelheim). While uptake of the vaccine has been relatively high in Australian herds (30%), many producers do not have the confidence to remove all antibiotics, as they have no way of knowing what percentage of their herd is protected from ileitis. Practitioners have also experienced vaccine failures in ecoshed housed pigs, which has forced them to re-introduce medication to help control ileitis.

Diagnosis of ileitis in live pigs has relied upon the detection of the bacteria *L. intracellularis* in pig faeces using a polymerase chain reaction (PCR) assay and/or detection of *L. intracellularis*-specific antibodies in sera using an immunoflourescent antibody test (IFAT) or a commercial ELISA (Boehringer Ingelheim). The PCR is a sensitive and specific diagnostic assay that indicates current infection in pigs clinically and sub-clinically affected with ileitis. However, the conventional PCR is not quantitative; hence the severity of disease cannot be evaluated and few people investigate *L.intracellularis* infection in pigs without diarrhoea. Therefore production losses associated with sub-clinical infection are often underestimated. In addition, due to the high sensitivity of the PCR assay, detection of *L. intracellularis* by PCR doesn't prove that *L. intracellularis* caused the diarrhoea.

The serological assays (IFAT and ELISA) detect IgG antibodies to *L. intracellularis*, and are a useful measure of the prevalence of clinical and sub-clinical *L. intracellularis* infections in a herd. Antibodies develop about 3 weeks after infection, and are therefore not a real time indicator of ileitis in pigs. In addition, blood is a more difficult and costly sample to collect than faeces. Both of these blood tests require specialist laboratories for the detection of the antigen-antibody complexes (ELISA plate reader or UV microscope).

Discussions with pig producers and veterinarians identified the need to develop a semi-quantitative assay to determine the bacterial 'load' of *L. intracellularis* in pigs and in the environment, and relate this to the efficacy of current control strategies (vaccination, medication or improved hygiene). To be useful, an alternative assay needed to be inexpensive, rapid to perform, semi-quantitative, sensitive and specific for *L. intracellularis*, able to demonstrate active infection and simple enough to be performed in the piggery or in a rudimentary laboratory. Veterinarians also wanted an assay that could quantify the risk of ileitis outbreaks if antibiotics were removed from feed.

2. Methodology

PART A: DEVELOPMENT OF AN ON-FARM FAECAL IMMUNOASSAY

Evaluate a range of inexpensive reagents and procedures to partially purify faecal samples for analysis.

Evaluate the sensitivity and specificity of currently available antibodies for the detection of *L. intracellularis* in faecal samples. Develop new polyclonal or monoclonal antibodies to *L. intracellularis* proteins.

Investigate a 'dipstick' immunoassay for the detection of *L. intracellularis* in faecal samples.

Investigate antibody coated magnetic beads to capture *L. intracellularis* from faeces.

Investigate lateral flow membranes that utilise specific antibodies to capture and detect *L. intracellularis* in faeces.

PART B: DEVELOPMENT OF A QUANTITATIVE PCR

If none of the above methods are sufficiently sensitive, develop a real time polymerase chain reaction assay to detect *L. intracellularis* in faeces.
Evaluate sensitivity and specificity of the new assays to detect *L. intracellularis* in the faeces of clinically affected pigs.

PART A: DEVELOPMENT OF AN ON-FARM FAECAL IMMUNOASSAY

Background

Prior to starting this work, we studied a range of platforms for a field based immunological assay for detecting *L.intracellularis* in pig faeces (outlined in appendix A). We selected the lateral flow immunoassay (LFIA) to provide a quick, qualitative response to the presence or absence of *L.intracellularis*. The signal is designed to be easily visualised and interpreted, and they do not require specialised equipment in their set up or analysis of results, such as incubators, electricity or refrigeration. We focussed on producing an assay that was robust and avoided hazardous chemicals. LFI assays can be produced comparatively cheaply in bulk and are designed to be used away from the laboratory, such as on-farm. Pregnancy tests are the most commonly known lateral flow immunoassay.

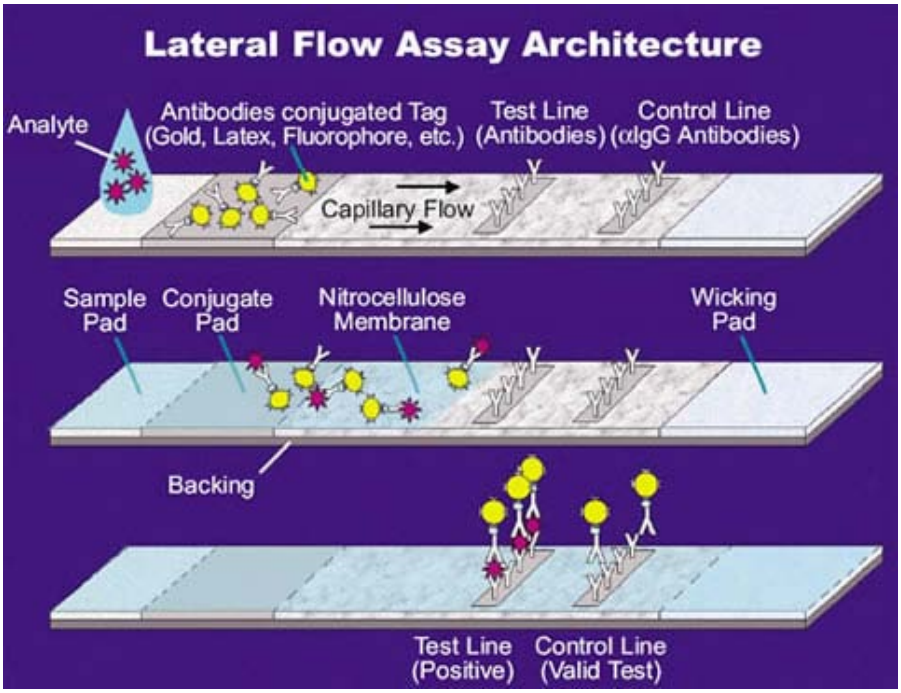

Figure 4.1 - Diagram of nitrocellulose lateral flow assay; the sample containing *L.intracellularis* (analyte) is applied to the sample pad (top image); the bacteria binds to the antibody coated beads on the conjugate pad and moves along the nitrocellulose membrane (middle image); the bead-antibody-bacteria complex is captured at the test line by anti-Lawsonia antibodies and excess beads move to the control line to be bound there (bottom image) (Guardian Biosciences, 2004)

We investigated two different lateral flow immunoassay (LFIA) systems to identify the most robust and specific assay for ileitis in the field. The first *L.intracellularis* LFIA used a hydrophobic nitrocellulose membrane (Millipore HiFlow 120) that binds proteins such as antibodies (Figure 4.1). Both the blue microspheres coated with rabbit anti-*L.intracellularis* IgG (R97) and the capture antibodies (anti-Lawsonia IgG and goat anti rabbit IgG) are bound to the nitrocellulose. Faecal suspensions are applied to the sample pad and drawn by capillary action to the conjugate pad, where *L.intracellularis* bind to R97 IgG coated microspheres (blue). The bead-antibody- antigen complex moves along the nitrocellulose membrane by capillary action to the test and control lines. Rabbit anti-Lawsonia IgG immobilised on the test line captures the *L.intracellularis*-antibody-bead complex to form a coloured line (Figure 4.1). Beads that have not bound *L.intracellularis* continue to move past the test line to the control line and are captured by the goat anti-

rabbit IgG, forming a second blue line. The wicking pad on the right side of the strip draws the sample along the membrane in one direction. Every faecal sample must be positive at the control line to ensure that each test has worked; this is the internal control for all test strips. Negative faecal samples don't bind to beads, but both beads and faeces move past the test line to the control line, and are bound to anti-rabbit IgG.

The second assay, known as 'boulders in the stream' is designed to avoid problems with protein-coated beads sticking to the membrane non-specifically, by using an inert membrane. We chose a hydrophilic membrane (Whatman Fusion 5) with pore sizes large enough for the blue microspheres coated with antibody (0.2µm) to move through the membrane (the stream), but the large beads (2.4µm boulders) coated with capture antibodies were immobilised on the membrane due to their size (Figure 4.2). This differs from the HiFlow 120 nitrocellulose membrane, which immobilises antibodies directly onto the membrane. Purified *L.intracellularis* antibodies are bound to small coloured latex beads and applied to the Fusion 5 membrane. Faecal suspensions (containing *L.intracellularis*) are applied to the membrane and bind to the rabbit anti-Lawsonia (R97) IgG-coated blue microspheres. The bead-antibody-antigen complex continues to move along the membrane by capillary flow until it is captured by immobilised white beads coated with R97 IgG (boulders) at the test line. Blue microspheres that don't bind *L.intracellularis* move to the control line, where they are captured by goat anti-rabbit IgG coated on the white beads. A positive result is indicated by a blue line. Like the previous test, every faecal sample must be positive at the control line to ensure that each test has worked.

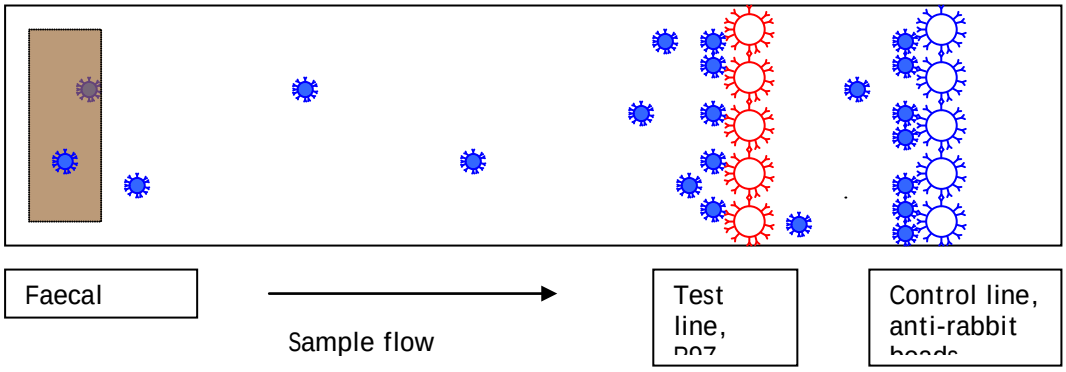

Figure 4.2 – "Boulders in the stream' diagram

Develop a lateral flow immunoassay to detect L. intracellularis in pig faeces

Evaluation of methods for faecal purification and solubilisation

Methods to prepare positive and negative controls

Large pools of known *L. intracellularis* negative and positive faeces (confirmed by conventional PCR) were prepared to evaluate faecal purification methods. Faeces from scouring pigs with ileitis were used to prepare a clinical pool (approximately 2 x 10^6 *L. intracellularis* /mL faecal supernatant) and faeces from pigs with sub-clinical ileitis (not scouring) were used to prepare a sub-clinical pool (2 x 10^4 *L. intracellularis*/mL). Dilutions of the positive pools were made in the negative faeces to mimic the detection of 10%, 20%, 40%, 60% or 100% positive pigs in a pool of faeces (ie. 10% equals detection of 1 positive pig in a pool of 10 samples).

Faecal sampling methods from commercial herds

Pooled pen faecal samples were collected from 4 commercial herds (Table 4.1) according to a protocol determined by the statistical program 'Pooled prevalence calculator' (http://www.ausvet.com.au/pprev/content.php?page=home). The pool size (number of samples per pool) and number of pools to be collected was calculated to ensure that the assay could estimate less than 10% prevalence of disease in individual animals from pooled samples (test sensitivity = 0.9 and specificity = 1.0). Generally between 3 and 8 faecal samples were randomly collected from the floor of pens or ecosheds and mixed by hand before pooling and at least 10 pools (and up to 40 pools) were collected per herd. Sera were also collected from herds 1 and 4 to validate the prevalence and severity of ileitis.

Table 4.1 Description of commercial herds for faecal sampling

Herd #	Housing	Production flow	Ileitis control
1	Ecoshed	AIAO	None
2	Conventional pens	AIAO	Antibiotics for SD eradication
3	Conventional pens	Continuous	Sporadic PHE
4	Ecoshed	AIAO	Antibiotics + sporadic PHE

Purification methods for faecal samples

Faecal samples were diluted in phosphate buffered saline (PBS) and partially purified by one of three methods prior to *L. intracellularis* detection by conventional PCR. The purification methods were; low speed centrifugation, filtration through a glass wool and sand column, or centrifugation through a commercial faecal parasite concentrator, able to separate fat and large debris (> 425µM) from faecal samples. DNA was extracted from the purified faecal samples with a Wizard® PCR Preps DNA Purification System (Cat No A7181, Promega, Madison, WI, USA) and amplified with a conventional PCR as previously described (Collins and Love, 2007).

Extraction and solubilisation methods for *L.intracellularis* from faeces

A range of detergents and mechanical disruption methods were used in the faecal extraction process to aid in the release or solubilisation of *L.intracellularis* proteins. Initially the non-ionic detergents Tween 20, Triton X-100 and Brij-58 were tested because they were expected to solubilise proteins without affecting the charge or biological activity of the bacterial proteins. Detergents were tested at a range of concentrations (0.025% to 0.1% w/v diluted in PBS), high enough to aid in protein release, but not too high to cause displacement of the capture antibodies on the test and control lines (Millipore, 2002).

Two mechanical disruption techniques were also tested for the release of *L.intracellularis* proteins. Glass beads (106µm) and zironcium beads (106µm) were mixed with faecal suspensions (equal weight to volume) by vortexing, then allowed to settle before supernatants were applied to lateral flow strips. Horse serum (10% v/v) was also added to the faecal sample buffer to prevent non-specific adsorption of R97-coated beads and *L.intracellularis* antigen to the nitrocellulose.

Evaluate the sensitivity and specificity of currently available antibodies or develop new antibodies for *L. intracellularis*

Monoclonal antibodies to *L.intracellularis* were sourced from a UK laboratory (IG4) and a pharmaceutical company (VPM53). While significant quantities of the monoclonal VPM53 could be obtained, it was less sensitive than the other monoclonal and would add substantially to the final cost of the assay, so we chose to develop our own polyclonal antibody.

Preparation and purification of a polyclonal rabbit anti-Lawsonia antibody

Rabbits were hyper-immunized with approximately 5 x 10^7 *L.intracellularis*, extracted from the mucosa of a PHE-affected pig and partially purified by filtration. Blood was collected after the fifth immunization to determine the concentration of antibodies to

L.intracellularis. The end point titre of the rabbit anti-Lawsonia sera (R97) was determined in a fluorescent faecal immunoassay (FFIA). Serial dilutions of rabbit sera were incubated with faecal smears from a PHE-affected pig shedding approximately 1 x 10^7 *L.intracellularis* per gram of faeces, and detected with an anti-rabbit IgG fluorescent antibody. Dilutions of the pre-immunization serum were also incubated with faecal smears to determine the minimum dilution of sera to avoid non-specific antigen-antibody binding.

An affinity chromatography column of Protein G resin (GenScript, #L00209) was prepared to purify the rabbit R97 sera. This recombinant protein G resin binds specifically to mammalian IgGs, removing any IgA, IgM and IgD from the sample. The rabbit anti-Lawsonia sera (R97) was diluted, filtered and then bound to the column, and any proteins other than IgG were removed by extensive washing of the resin with PBS pH 7.5 (± 0.5M NaCl, ± 0.05% Tween 20). Rabbit IgGs were then eluted in 100mM citric acid, pH 3, and 0.5mL fractions were collected and buffered in an equal volume of 0.5M Na phosphate. Approximately 20 protein fractions were collected and analysed by polyacrylamide gel electrophoresis (PAGE) to identify fractions containing rabbit IgG. Reduced (0.25M Tris-HCl, pH 6.8, 40% glycerol, 1.6% w/v SDS, 0.1% bromophenol blue and 0.5% ß-mercaptoethanol) or non-reduced (without ß-mercaptoethanol) loading dye was added to alternate fractions and samples boiled for 5 mins before loading onto the 12% acrylamide gel. Molecular weight markers (Fermentas Page Ruler, #SM0671) were loaded onto the same gels to give an indication of protein sizes. All proteins were then stained with coomasie blue (BioRad, Gel Code Blue).

Fractions containing IgG were pooled and concentrated on a Viva spin 20 column (Sartorius Stedim #VS2041), then dialysed in PBS for 2 days to remove excess salts. The protein concentration was determined with a BCA Protein assay (Pierce, #23225) using BSA standards between 0 and 1mg/mL, and an optical density of 562nm.

Sensitivity and specificity of R97 rabbit-anti-Lawsonia sera

Faeces collected from *L.intracellularis* PCR-positive (n=30) or PCR-negative pigs (n=33) were used to validate the sensitivity and specificity of the R97 rabbit-anti-*L.intracellularis* sera. Faecal smears from these pigs were incubated with the R97 rabbit sera and detected with a fluorescently labelled anti-rabbit IgG antibody (FFIA). The number of *L.intracellularis* per gram of faeces was determined for each sample by counting the mean number of *L.intracellularis* in 10 field diameters viewed under 1000x magnification with the UV microscope. The proportion of positive FFIA and faecal PCR results were compared, and the number of *L.intracellularis* per gram of faeces (FFIA) was correlated with the clinical scores of diarrhoea for these pigs on the same day.

The sensitivity and specificity of the R97 polyclonal sera was further confirmed by immunohistochemical (IHC) staining of formalin fixed pig ileal sections that had previously stained IG4 positive or negative for *L.intracellularis.* Positive staining with the *L.intracellularis* specific IG4 monoclonal antibody is the gold standard for the diagnosis of proliferative enteropathy. Sections of unstained tissue for IHC were dewaxed and endogenous peroxidase was blocked prior to trypsin digestion of tissues. Non-specific antibody binding was prevented by incubation with goat serum, and then tissues were incubated with either the IG4 monoclonal or the R97 rabbit anti-*L.intracellularis* sera (both diluted 1:200 in PBS). Tissues were washed in PBS then incubated with either Envision goat-anti-mouse IgG HRP or goat-anti-rabbit IgG HRP. The antigen-antibody complex was visualized by staining with the chromagen aminoethyl carbazol, and intestinal tissue was counterstained with Mayer's haematoxylin.

5.1.3 Characterisation of immunoreactive proteins

Proteins were extracted from a number of *L.intracellularis* isolates and separated on polyacrylamide gels. Similar quantities of protein preparations were loaded onto gels and then transferred to membranes for reaction with either the anti-*L.intracellularis* monoclonal antibodies (IG4 and VPM53) or our rabbit anti- *L.intracellularis* sera (R97).

Proteins were also extracted from our positive and negative faecal pools and separated on polyacrylamide gels. Proteins were transferred to membranes and reacted with either the anti-*L.intracellularis* monoclonal antibodies (IG4 and VPM53) or our R97 rabbit anti-*L.intracellularis* sera.

Cross-reactivity of rabbit anti-Lawsonia sera

The absence of cross-reactivity with other pig pathogens was tested in immunoassays with pure cultures of a wide range of enteric bacteria. Cultures of *Salmonella typhimurium, S.choleraesuis, Brachyspira pilosicoli, B.hyodysenteriae, Campylobacter fetus fetus, Desulfovibrio desulfuricans, Yersinia enterocolitica, Clostridium perfringens,* and porcine strains of *E.coli* (0149, 0157 and 0141) were fixed on glass slides and incubated with our R97 rabbit anti-*L.intracellularis* sera. Antigen-antibody interactions were detected with a FITC conjugated goat-anti-rabbit IgG secondary antibody and viewed with a UV microscope.

Absorption of non-specific pig proteins from rabbit R97 sera

The rabbit was immunised with partially purified *L.intracellularis* extracted from ileitis affected pig intestine. The obligate intracellular nature of *L.intracellularis* makes it very difficult to produce a pure culture of *L.intracellularis* to immunise rabbits with. It was therefore possible that the immunised rabbit produced IgG antibodies to proteins other than *L.intracellularis*, and these could cross-react with proteins in pig faeces. To overcome this potential problem, the R97 rabbit anti-*L.intracellularis* sera was purified by absorbing out any antibodies raised against other proteins present in the intestinal cells of healthy pigs. Intestinal cells were extracted from healthy pigs and partly purified before mechanical lysis. The lysed cells were separated into soluble and insoluble fractions by centrifugation and then mixed overnight with our R97 rabbit sera. The specificity of the absorbed R97 rabbit sera (R97-5 for insoluble proteins and R97-6 for soluble proteins) was tested in a western blot of pig faeces from *L.intracellularis* positive and negative pigs.

Affinity purification of the R97 rabbit anti-*L.intracellularis* sera

An affinity purification column was prepared to remove all non-*L.intracellularis* antibodies from the R97 rabbit sera, with the aim of overcoming the non-specific binding of R97-coated beads to the lateral flow test line in *L.intracellularis* negative pig faeces. Proteins extracted from purified *L.intracellularis* (sonication with 0.1% Triton X-100) were bound to a commercial matrix and the R97 rabbit serum was passed through the *L.intracellularis* affinity column. Only antibodies specific to *L.intracellularis* should bind to the column and all non-*L.intracellularis* antibodies in the R97 serum should be washed off the column. The purified R97 rabbit anti-*L.intracellularis* antibodies are then eluted from the column. A second attempt was made to bind extracted *L.intracellularis* proteins (sonicated with 1% octyl-ß-glucoside, a non-ionic detergent) with a new matrix (Ultralink Biosupport). R97 rabbit sera purification was also attempted with this second affinity column.

Pilot study to assess the *L.intracellularis* qRT PCR assay to monitor ileitis control in pigs experimentally challenged with *L.intracellularis*

Faecal samples from earlier experimental pig trials investigating the control of ileitis were re-tested with the *L.intracellularis* qPCR. In the first study, groups of 5 naive pigs were medicated continuously in-feed with oxytetracycline (OTC) at 50 and 100ppm, 50ppm Tylan or non-medicated for four days before experimental challenge with *L. intracellularis*. Pigs were weighed weekly and faeces and blood were collected 1-2 times per week from each pig. In the second study, groups of 5 pigs were medicated with

CTC or OTC at 400ppm or Tiamulin at 200ppm in-feed 2 weeks after experimental challenge with *L. intracellularis*. Pigs were weighed weekly and faecal and blood samples were collected as described above.
A third study investigated the effect of vaccination with Enterisol® Ileitis on the control of clinical disease and faecal shedding of *L.intracellularis*. One group of 30 pigs was challenged with virulent *L.intracellularis* 6 weeks after vaccination, and the other group (n=30) were challenged, but were not vaccinated. Pigs were weighed weekly and blood and faeces was collected 1-2 times per week. DNA was extracted from faeces and the number of *L.intracellularis* per gram of pig faeces was determined with the *L.intracellularis* qPCR.

Monitoring *L.intracellularis* infection in wild rodents trapped on pig farms

Rats were either shot or trapped on three Australian pig farms located in the State of Victoria (farms A, B and C) and one in South Australia (farm D) over a two to eight week period. Twenty-eight rats were trapped at Farm A, 120 were obtained from each of farm B and farm D and 60 were shot on farm C. Rats were stored at -20°C prior to dissection of the intestine and extraction of DNA. The intestinal mucosa was scraped with the blade of a sterile scalpel to obtain about 0.2 to 0.3 g of both tissue and faeces. DNA was extracted from each sample and the number of *L. intracellularis* per gram of faeces and mucosa was determined with the *L.intracellularis* qPCR (Collins et al., 2011).

3. Results

Evaluation of methods for faecal purification and solubilisation

Comparison of faecal purification methods for known positive and negative samples and field samples

Faecal samples partly purified by either the glass wool and sand filtration method or the Parasep separator columns increased the sensitivity of conventional PCR compared with low speed centrifugation or no purification (Table 4.2). The dilution of the faecal suspension appeared to be important with optimal sensitivity at a dilution of 1:5 (w/v) with all clean up methods.

Table 4.2 - Comparison of different faecal preparation methods for PCR detection of *L. intracellularis* from pooled faecal samples collected from herd 4.

Faecal preparation method	Dilution of faeces	Prevalence of positive pools
None	1:5	25%
Low speed centrifugation	1:2	25%
Low speed centrifugation	1:5	50%
Glass wool + sand filtration	1:5	75%
Glass wool + sand filtration	1:10	50%
Parasep columns	1:2	25%
Parasep columns	1:5	75%

The glass wool and sand filtration method also increased the PCR sensitivity in pools of positive faeces prepared from pigs clinical and subclinically affected with ileitis (Table 4.3). The optimal dilution of the faecal suspension also appeared to be 1:5 for our clinical and subclinical ileitis faecal pools. The glass wool clean up of the 1:5 faecal suspension increased the detection sensitivity of the PCR to be able to detect 1 positive clinical pig in a pool with 9 negative pigs and 4 positive subclinical pigs in a pool with 6 negative pigs. This was a significant improvement on the previous method without a preliminary clean up step, especially when detecting *L. intracellularis* in faecal pools with subclinically affected pigs.

Table 4.3 - Comparison of glass wool and sand filtration (GWS) with no faecal clean up for PCR detection of *L. intracellularis* from pools of faeces with clinical and subclinical ileitis

Positive pool	Proportion of positives in pool	No clean up & PCR	GWS & PCR 1:5 w/v	GWS & PCR 1:10 w/v
Clinical	100%	+	+	+
Clinical	60%	+	+	+
Clinical	40%	+	+	+
Clinical	20%	+	+	-
Clinical	10%	-	+	-
Negative	0%	-	-	-
Sub-clinical	100%	+	+	-
Sub-clinical	60%	-	+	-
Sub-clinical	40%	-	+	-
Sub-clinical	20%	-	-	-
Sub-clinical	10%	-	-	-
Negative	0%	-	-	-

L. intracellularis were more clearly visible in faeces purified with the glass wool and sand filtration method compared with the Parasep column when stained with the monoclonal antibody IG4 (McOrist et al., 1987). Although the Parasep columns were expected to remove large particles and fat from faecal samples, there was still significant background material which made visualisation of *L. intracellularis* difficult.

The proportion of positive faecal pools correlated well with the prevalence of seropositive pigs in herd 4, with no evidence of *L.intracellularis* infection in herd 1 regardless of the diagnostic assay used (Table 4.4).

Table 4.4 - Prevalence of *L. intracellularis* (LI) infection in herds as estimated by PCR from pooled faecal samples (glass wool + sand filtration) and serology (IFAT)

Herd #	Housing	Age of pigs	Clinical ileitis	Prevalence of LI antibodies	Prevalence of LI infection (PCR)
1	Ecoshed	6	No	0%	0%
	Ecoshed	8	No	0%	0%
	Ecoshed	10	No	0%	0%
	Ecoshed	12	No	0%	0%
	Ecoshed	14	No	0%	0%
	Ecoshed	16	No	0%	0%
	Ecoshed	18	No	0%	0%
2	Concrete pens	10	No		0%
	Concrete pens	12	No		0%
	Concrete pens	14	No		0%
	Concrete pens	16	No		0%
	Concrete pens	18	No		0%
3	Concrete pens	9	No		0%
	Concrete pens	11	No		0%
	Concrete pens	13	No		50%
	Concrete pens	15	Yes		100%
	Concrete pens	17	Yes		100%
	Concrete pens	19	No		0%
4	Ecoshed	9	No	0%	0%
	Ecoshed	10	No		0%
	Ecoshed	11	No		0%
	Ecoshed	12	No		0%
	Ecoshed	13	No		0%
	Ecoshed	14	No	30%	25%

Herd #	Housing	Age of pigs	Clinical ileitis	Prevalence of LI antibodies	Prevalence of LI infection (PCR)
	Ecoshed	15	No		0%
	Ecoshed	16	No		0%
	Ecoshed	17	No		25%
	Ecoshed	18	Yes	80%	75%

Extraction and solubilisation of *L.intracellularis* from faeces

Similar concentrations of protein were extracted from *L.intracellularis* positive faeces solubilised in 0.1% Triton X-100, 0.05% Tween 20 or 0.1% Brij 85, with or without mechanical disruption (glass or zirconium beads). *L.intracellularis* proteins solubilised in the above detergents were also able to bind to R97 antibody-coated beads in a fluorescent immunoassay (viewed under UV microscope). However, the effect of detergents on protein solubilisation was less clear when faeces were tested on lateral flow immunoassay strips.

L.intracellularis solubilised in 0.05% Brij 58 produced a strong control line (goat anti-rabbit IgG), but no test line (rabbit anti-*L.intracellularis* IgG) for positive or negative faeces (Table 4.5), indicating that while Brij 58 solubilised proteins may have bound to R97 beads, they were not captured by the R97 antibody on the test line. Some detergents can displace the capture antibodies, preventing the formation of a positive test line (Millipore 2002). Increasing the concentration of Brij 58 caused problems for bead flow along the membrane. Mechanical lysis of faecal proteins with glass or zirconium beads did not alter the above results.

Table 4.5 - The effect of detergents and mechanical lysis on LFIA test results from *L.intracellularis* positive (100% 2131) and negative (0%, 2132) faecal samples

Faecal extraction buffer	Mechanical lysis	Test/control line results (positive faeces)	Test/control line results (negative faeces)
0.05% Brij 58	No	-/+	-/+
0.1% Brij 58	Yes	-/+	-/+
0.2% Brij 58	No	DR	DR
0.05% Tween 20	No	+/+	+/+
0.1% Tween 20	No	DR	DR
0.05% Tween 20	Yes	+/+	+/+
0.2% TX-100	No	-/-	-/-
0.1% TX-100	No	+/+	-/+
0.05% TX-100	No	+/+	+/+

DR = LFIA did not run properly

L.intracellularis solubilised in 0.05% Tween 20 produced a very weak test line and a strong control line, with both the negative and positive faeces. Increasing the Tween 20 to 0.1% didn't improve the specificity or sensitivity of the reaction, because the extra Tween 20 hindered the flow of the R97-coated beads. Combining mechanical disruption with Tween 20 did not improve the specificity or sensitivity of the result.

Solubilising proteins in 0.2% Triton X-100 appeared to displace the capture antibodies on both the test and control lines, with beads moving past both lines. However, *L.intracellularis* solubilised in 0.05% Triton X-100 didn't form a test or control line. Triton X-100 at 0.1% appeared to produce the most reliable and specific test result, producing a weak test line and strong control line for positive faeces, with negative faeces producing no test line and a strong control line.

Decision Point

L. intracellularis was detected by conventional PCR more consistently in pooled faecal samples after partial purification through a glass wool and sand filter. As this method is also inexpensive and easy to perform on farm, we continued with this method for all

future experiments. Faecal solubilisation methods that worked well for fluorescent antibody assays or PCR didn't work well for the LFIA. The LFIA was most specific when 0.1% Triton X-100 was added to positive and negative faeces prior to application to the test strip. While the addition of Triton X-100 improved the LFIA specificity, the assay sensitivity and specificity could be further improved in the commercialisation phase of the project. As mechanical disruption did little to improve the sensitivity of the assay, we did not continue with it, due to the difficulty of reproducing these methods in the field.

Antibody titre of R97 rabbit-anti-Lawsonia sera

The rabbit anti-Lawsonia serum (R97) was able to detect *L.intracellularis* proteins in faecal immunoassays, ileal tissue by immunohistochemistry and separated proteins by Western blots. The R97 sera was more concentrated than either monoclonal in all of the assays (Table 5.1).

Table 5.1 - Optimal dilutions of anti-*L.intracellularis* antibodies for immunoassays

Immunoassay	Monoclonal IG4	VPM53	Rabbit R97
Fluorescent faecal immunoassay	1:200	nt	1:4000
Immunohistochemistry	1:200	1:200	1:200 -1:400
Western Blot	1:2000	1:1000	1:4000-1:8000

Following purification on a protein G column, the R97 sera contained two distinct proteins at 50kDa and 25kDa, corresponding to the heavy and light chains of IgG respectively (Figure 5.1). Protein G purified fractions not reduced with ß-mercaptoethanol appeared as a single 150kDa protein, corresponding to intact IgG (2 light chains and 2 heavy chains).

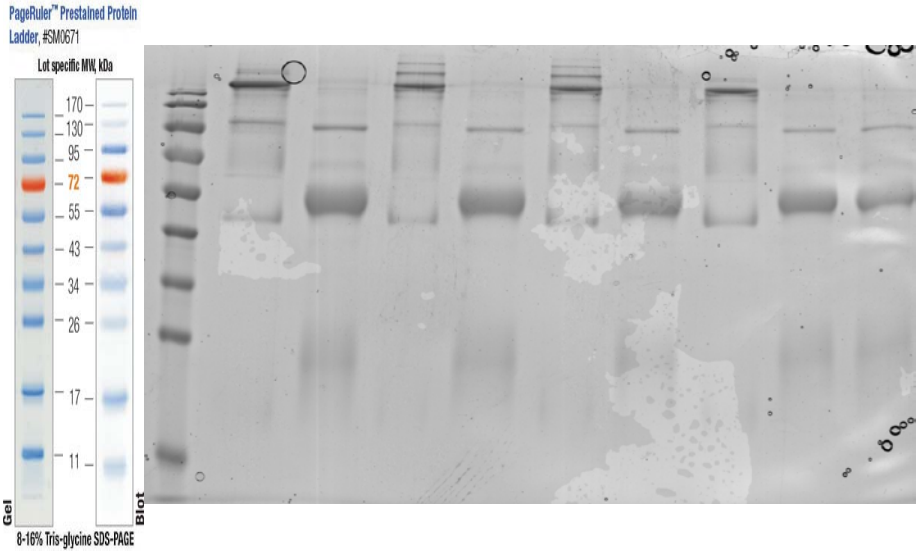

Figure 5.1 - Protein G purification of R97 rabbit sera: Lane 1: molecular weight markers, lanes 2, 4, 6 and 8 are non-reduced fractions, lanes 3, 5, 7, 9 and 10 reduced fractions.

Sensitivity and specificity of rabbit-anti-Lawsonia sera R97

The rabbit anti-*L.intracellularis* sera R97 was able to detect between 7 x 10^4 and 1 x 10^8 *L.intracellularis* per gram of faeces by fluorescent immunoassay (FFIA) in clinical and sub-clinically infected pigs. The number of *L.intracellularis* detected by FFIA with the R97 rabbit antisera was highly correlated with clinical signs of disease on the same day (r = 0.89). Although the conventional PCR was more sensitive (2 x 10^3 Li/g faeces), the FFIA was able to detect nearly 70% of the PCR positive samples (Table 5.2).

Table 5.2 - Comparison of PCR and fluorescent faecal immunoassay for detection of *L.intracellularis* shedding in pig faeces

	# of samples	% PCR positive	% FIA Positive
Known negatives	30	0%	0%
Known positives	33	100%	69.7%

The R97 rabbit-anti-*L.intracellularis* sera was able to detect *L.intracellularis* in all tissues previously shown to be IHC positive for *L.intracellularis* using the IG4 monoclonal antibody (gold standard for diagnosis of PE), and did not lead to any non-specific binding or false positive results (Table 5.3).

Table 5.3 - Immunohistochemical detection of *L.intracellularis* using R97 rabbit antisera in known IG4 positive and negative intestinal tissue

Tissue ID	Pathology	IHC with IG4	IHC with R97 sera
1164/91	PHE outbreak	++	++
5722/92	PHE outbreak	++	++
5333/93	PIA outbreak	+	+
275/99-1	PHE outbreak	+++	+++
275/99-2	PHE outbreak	++	++
278/99-2	Experimental PHE	+++	+++
1323/99-2	Experimental PIA	++	++
9089/06- 10	Experimental PHE	+++	+++
9089/06- 11	Experimental PHE	+++	+++
9089/06-22	Uninfected control	-	-
9089/06-23	Uninfected control	-	-
4493/09-13	PHE outbreak	+++	+++
4493/09-16	PHE outbreak	+++	+++

Characterisation of immunoreactive proteins

All three antibodies (the two monoclonals IG4 and VPM53 and our rabbit anti-*L.intracellularis* R97 sera) reacted with the same 22kDa protein from the porcine *L.intracellularis* isolate given as inoculum to the rabbit. Of the 4 other porcine isolates of *L.intracellularis,* only the R97 sera reacted with the same 22kDa protein in each preparation (Table 5.4). Initially we believed this was due to the higher sensitivity of the rabbit anti-*L.intracellularis* sera compared with the *L.intracellularis* monoclonal antibodies. However, later work suggested that the R97 sera could react with both denatured and native forms of *L.intracellularis* proteins, whereas the monoclonal antibodies could only react with non-denatured proteins. As our PAGE and Western blot were performed under denaturing conditions, it is not surprising that the IG4 and VPM53 monoclonal did not react with all the *L.intracellularis* isolates.

Table 5.4 - Western blot analysis of immunoreactive proteins in *L.intracellularis*

Lawsonia isolate ID	Source of Lawsonia	Reaction with IG4 monoclonal	Reaction with VPM53 monoclonal	Reaction with R97 rabbit sera
Rabbit inoculum	PHE outbreak	+++	++	+++
2189/94	PHE outbreak	+	±	+
799/03	PHE outbreak	±	-	++
9089/06	Experimental PHE	-	-	+
7423/07	Experimental PHE	-	-	+

± weak reaction

Cross-reactivity of R97 rabbit anti-*L.intracellularis* sera

The R97 rabbit antisera did not cross-react with any of the pig enteric pathogens tested, including Salmonella typhimurium, S.choleraesuis, Brachyspira pilosicoli, B.hyodysenteriae, Campylobacter fetus fetus, Desulfovibrio desulfuricans, Yersinia enterocolitica, Clostridium perfringens, and porcine strains of E.coli (0149, 0157 and 0141).

The R97 rabbit anti-*L.intracellularis* sera reacted with a number of proteins in the faeces of *L.intracellularis* positive and negative pigs (Western blot), and also reacted with a high proportion of *L.intracellularis*-negative faecal samples in the lateral flow immunoassay. R97 sera absorbed against both the insoluble (R97-5) and soluble (R97-6) fractions of healthy pig gut cells retained specific antibodies to *L.intracellularis* (visible at 22kDa), but absorption with either fraction did not remove all non-specific antibodies in the rabbit sera that cross-react with healthy pig faeces (Figure 5.2).

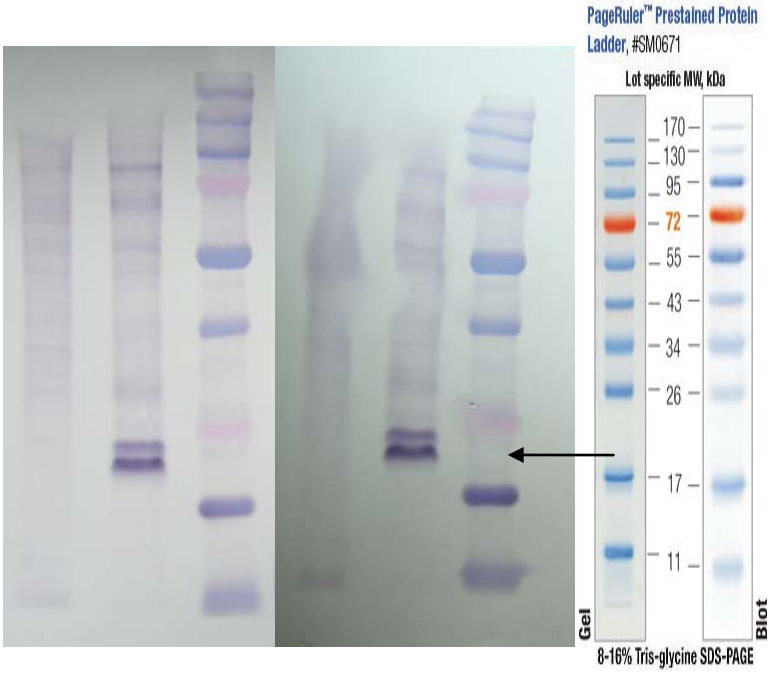

Figure 5.2 Western blot of *L.intracellularis* positive (lanes 2 and 5) and negative (lanes 1 and 4) faeces reacted with R97-5 sera absorbed against soluble healthy pig enterocytes (lanes 1-3) or R97-6 sera adsorbed against insoluble pig enterocytes (lanes 4-6) with molecular weight markers in lanes 3 and 6. Arrow indicates immunoreactive 22kDa protein of *L.intracellularis*.

Affinity purification of R97 rabbit anti-*L.intracellularis* sera

The initial attempt to prepare an *L.intracellularis* affinity chromatography column was not successful. We had difficulty solubilising and binding enough *L.intracellularis* proteins to the matrix, leading to poor recovery of purified antibodies from the column. *L.intracellularis* proteins solubilised with 1% octyl-ß-glucoside, and bound to the Ultralink Biosupport matrix were able to capture R97 rabbit anti-Lawsonia IgGs. However, we are yet to test whether these purified antibodies will overcome non-specific reactions in the lateral flow immunoassay.

5.3 Conclusion

4. 6.0 Adsorbing R97 anti-*L.intracellularis* IgG to latex beads

6.1 Methods:

Antibodies can be coated or adsorbed onto the surface of polystyrene microspheres (beads) due to hydrophobic interactions between the beads and the hydrophobic portions of the antibody. This leaves the Fab region of the antibody (antigen binding site) available to bind *L.intracellularis* antigen. However, antibodies can be bound in the incorrect orientation if insufficient antibody is bound to the bead; therefore saturation of the bead with antibody needs to be achieved (Bangs, 2008c). The manufacturers recommend 3 to 10 times saturation, which can be calculated with the formula below:

$$S = (6/\rho D)(C),$$

where S = the amount of protein needed for surface saturation (mg protein/g beads), C = capacity of the bead surface for protein = 2.5 mg protein/m^2 beads for bovine IgG, and $6/\rho D$ = surface area/mass (m^2/g) for beads of a given diameter (ρ = density of beads = 1.05g/cm2 for polystyrene) and D = diameter of beads in microns).

Saturation of 1mg of 0.21µm blue polystyrene microspheres (Bangs Laboratories, DS02B) therefore required 71.4mg of R97 IgG per gram of beads. Saturation of 1mg of the 2.4µm white polystyrene beads (Bangs Laboratories, PS05N) with goat anti-rabbit IgG (Sigma R5506) required 5.95 mg of antibody per gram of beads. Surface saturation of both beads was tested with 2 to 10 times saturation. Antibodies were adsorbed onto beads by mixing at 4°C overnight in a range of adsorption buffers (Table 6.1, Bangs 2008c), and then any remaining hydrophobic areas on the beads were blocked with 1% BSA. Beads were washed in bead wash buffer (10mM Na Phosphate, 0.8% NaCl, 0.05% Tween 20) and resuspended in bead storage buffer (100mM borate buffer, pH 8.2, with 0.1% BSA, 0.05% Tween 20 and 0.02% sodium azide) (Henderson and Stewart, 2000).

Table 6.1 Adsorption buffers for passive adsorption of R97 IgG to blue microspheres

Buffer	pH
Phosphate buffered saline	7.4
Borate buffer	8.5
Carbonate-bicarbonate buffer	9.45
Citrate phosphate buffer	6.6

Bangs Laboratories Technote 204 (Bangs 2008c).

The amount of antibody adsorbed on to beads was determined by incubating the R97 coated beads with 1/10,000 mouse anti-rabbit IgG conjugated to alkaline phosphatase (50:50 Sigma A2306 and A2556) for an hour with mixing. Beads were washed in bead wash buffer 3 times and then incubated with an alkaline phosphatase substrate (50:50 Sigma A9851 and A0227). The saturation of beads with antibodies was determined by comparing the optical density at 620nm on a spectrophotometer. Beads coated with 1% BSA were used as a negative control. The concentration of goat anti-rabbit IgG bound to large white latex beads was determined by incubating beads with 1/5000 rabbit anti-goat IgG conjugated to horse radish peroxidise (Sigma A5420), washed as above and then developed with ABTS (Zymed 00-2024) in citrate buffer and protein concentration measured at an absorbance of 405nm.

However, the above assay was not able to demonstrate whether the R97 IgG was bound in the correct orientation and able to capture *L.intracellularis* antigen. A second fluorescent immunoassay was developed to determine this. R97 IgG-coated beads were incubated with *L.intracellularis* for an hour with mixing, and then non-specific binding was removed with 3 washes in PBS, 0.05% Tween 20. Beads were then incubated with pig anti-Lawsonia IgG sera for an hour, washed again, and then incubated with rabbit anti-pig IgG conjugated to FITC (Sigma F1638) for an hour. After non-specific binding was removed with 3 washes,

the bead complex was dried on a slide and the presence of *L.intracellularis* bound to beads was observed with a UV microscope.

6.2 Results

Optimal adsorption of R97 IgG on the blue microspheres was achieved with a two times surface saturation of antibody. Increasing the amount of IgG up to 10 times saturation did not lead to increased concentrations of R97 IgG binding to the beads (Table 6.2). Optimal adsorption of R97 IgG on the large white beads was achieved at 3 times saturation and adsorption of goat anti-rabbit IgG to the large white beads was 5 times saturation (Table 6.3).

Table 6.2 Saturation of R97 IgG antibodies bound to blue latex beads, measured by absorbance at OD_{620}.

Surface saturation with R97 IgG	OD_{620}.
2 x	2.189
3.5 x	2.053
7x	1.980
BSA control	0.193

The quantity of R97 IgG bound to blue microspheres was not significantly affected by the adsorption buffer, with similar absorbance for all buffers (Table 6.4). The manufacturers recommended using a slightly basic buffer with a pH close to the pI of the protein (Bangs, 2008c). The ability of R97 IgG coated beads to bind *L.intracellularis* was determined in a fluorescent immunoassay. Fluorescing *L.intracellularis* attached to beads, regardless of which adsorption buffer was used to coat the R97 IgG to the blue microspheres (results not shown).

Table 6.3 Optimal adsorption of antibodies onto latex beads for the Fusion 5 and HiFlow 120 membranes

Membrane type	Optimal saturation on 0.2µm blue beads	Optimal saturation on 2.4µm white beads
Fusion 5	2 x R97 IgG	3 x R97 IgG
	2 x R97 IgG	5 x goat anti-rabbit IgG
Millipore HiFlow 120	2 x R97 IgG	N/A

Table 6.4 Optimal adsorption buffer for R97 IgG onto blue latex beads

Adsorption buffer	OD_{620}.
PBS, pH 7.4	2.581
Borate buffer, pH 8.5	2.825
Carb-bicarb buffer, pH 9.4	2.750
Citrate phosphate buffer, pH 6.6	2.898

5. 7.0 Assembling the lateral flow strips

7.1 Methods

The optimal concentration of capture antibodies at the test (rabbit anti-*L.intracellularis* IgG) and control line (goat anti-rabbit IgG) was determined by varying the concentration of antibodies between 1µg and 10µg, but maintaining the same concentration of R97-coated beads with positive (*L.intracellularis* bacteria) and negative (PBS) samples. Antibodies were dried onto the nitrocellulose at 45-50°C for 30 mins, and strips were stored in a vacuum desiccator to ensure the antibodies remained strongly bound to the nitrocellulose. Antibodies were diluted in PBS, to avoid excess salts which could form a salt bridge, reducing the antibody's ability to bind to the antigen (Millipore 2002).

Initial experiments were undertaken with non-blocked nitrocellulose membranes. However, a range of detergents and inert proteins were used to block membranes when non-specific reactions were observed at the test line with negative faeces (Table 7.1). Nitrocellulose membranes with bound capture antibodies were incubated in blocking solutions at room temperature for 30 mins, before excess solution was blotted and membranes were dried at 45-50°C for 30 minutes.

R97-coated beads were dried onto glass wool fibre pads, which had been pre-treated with 30% w/v sucrose to help rehydration of the beads when sample was applied (Bangs, 2008d). Lateral flow strips were assembled in the following order: capture antibodies were dried onto the membrane, R97-coated beads were applied to the conjugate pad (adjacent to nitrocellulose), the sample pad was placed on top of the conjugate pad, and the absorbent pad was placed at the bottom of the nitrocellulose to wick the sample along the strip (Figure 4.1).

Table 7.1 Blocking buffers tested for nitrocellulose membranes

ID	Buffer description
1	1%w/v poly vinyl alcohol (MW 9000-10,000) in PBS
2	0.5% w/v poly vinyl alcohol (MW 9000-10,000) in PBS
3	0.2% w/v poly vinyl alcohol (MW 9000-10,000) in PBS
4	0.05% w/v poly vinyl alcohol (MW 9000-10,000) in PBS
5	1% w/v skim milk in PBS
6	0.5% w/v skim milk in PBS
7	0.1% w/v skim milk in PBS
8	0.5% w/v polyethylene glycol 6000 + 0.05%v/v Tween 20 in Tris saline
9	0.1% w/v polyethylene glycol 6000 + 0.05%v/v Tween 20 in Tris saline
10	3% w/v gelatine in 0.05%v/v Tween 20 in Tris saline
11	1% w/v gelatine in Tris saline
12	0.5% w/v gelatine in 0.05%v/v Tween 20 in Tris saline
13	0.05%v/v Tween 20 in PBS
14	0.1%v/v Tween 20 in PBS
15	1% w/v bovine serum albumin + 0.05% v/v Tween 20 in Tris saline
16	0.25% w/v sucrose, 0.1% w/v skim milk, 0.002% v/v Tween 20 in PBS

The sample pad and conjugate pad were removed from lateral flow strips when we were comparing sample buffers (section 4.1.4). R97-coated beads were mixed with the sample buffers and put in a flat bottom well. The nitrocellulose strip was in direct contact with the buffer and beads.

7.2 Results

The optimal concentration of both the capture antibodies at the test (R97 rabbit anti-*L.intracellularis* IgG) and control line (goat anti-rabbit IgG) was between 8 and 9µg protein. The effect of a wide range of blocking solutions on the LFIA specificity was initially tested with a positive (2131, 100%) and negative (2132, 0%) faecal sample diluted in 0.05% v/v Tween 20 in PBS. It was difficult to identify a concentration of inert protein in any of these blocking buffers that overcame non-specific reactions at the test line for negative faeces, without also blocking the specific binding at the test line for positive faeces. For example, blocking membranes with 0.05% polyvinyl alcohol (PVA) didn't prevent the development of a positive test line with negative faeces. However, increasing the concentration of PVA led to negative test lines in both positive and negative faeces (Table 7.2). The same was true for skim milk powder, though 1% skim milk stopped the bead flow completely (DNR= did not run). Polyethylene glycol and gelatine blocked the test line for both positive and negative faeces regardless on the concentration used.

Table 7.2 LFIA results for positive and negative faeces on membranes blocked with a wide range of buffers.

	Positive faeces (2131, 100%)	Negative faeces (2132, 0%)
Buffer description	Test/control line	Test/control line
1% PVA, PBS	-/+	-/+
0.5% PVA, PBS	-/+	-/+
0.2% PVA, PBS	-/+	-/+
0.05% PVA, PBS	+/+	+/+
1% milk, PBS	DNR	DNR
0.5% milk, PBS	-/+	-/+
0.1% milk, PBS	-/+	-/+
0.5% PEG, 0.05% Tw20, Tris saline	-/+	-/+
0.1% PEG, 0.05% Tw20, Tris saline	-/+	-/+
3% gelatine, 0.05% Tw20, Tris saline	-/+	-/+
1% gelatine, Tris saline	-/+	-/+
0.5% gelatine, 0.05% Tw20, Tris saline	-/+	-/+
0.05% Tween 20, PBS	+/+	+/+
0.1%v/v Tween 20, PBS	DR	DR
1% BSA, 0.05% Tw20, Tris saline	+/+	+/+
0.25% sucrose, 0.1% milk, 0.002% Tw20	+/+	-/+

DNR= did not run

It became clear that we needed to block any non-specific reactions before the faecal sample was applied to the LFIA. Therefore, in the final experiments we used 0.1% Triton X-100 to solubilise faecal samples prior to addition to LFIA membranes blocked with 0.25% sucrose, 0.1% milk, 0.002% Tw20 in PBS to successfully increase the specificity of the LFIA. These conditions are yet to be evaluated with intact strips (beads embedded in Conjugate pad).

7.2 Summary

After significant trial and error, we believe we are close to finding the assay conditions that provide the specificity required for this assay. However, more test development is needed and this test needs to be validated with the 160 field samples collected for the *L.intracellularis* qPCR (section 8.1.3). The specificity of the LFIA may be improved by further purification of the R97 sera by affinity chromatography or by using a monoclonal antibody to coat the beads. This could be investigated in consultation with a commercial partner.

6. PART B: DEVELOPMENT OF A QUANTITATIVE PCR

7. 8.0 Development of a quantitative PCR for *L.intracellularis* in faeces

The key difference between real time PCR (RT PCR) and conventional PCR is that the amplified DNA is detected after each cycle (in real time), rather than waiting until the end of 30 cycles as required for conventional PCR. RT PCR can therefore amplify and simultaneously detect the target DNA. The procedure requires fluorescent labelling of sequence-specific probes, where the fluorescent marker (reporter) is cleaved and detected only after the probe hybridises to the amplified target DNA (Figure 8.1).

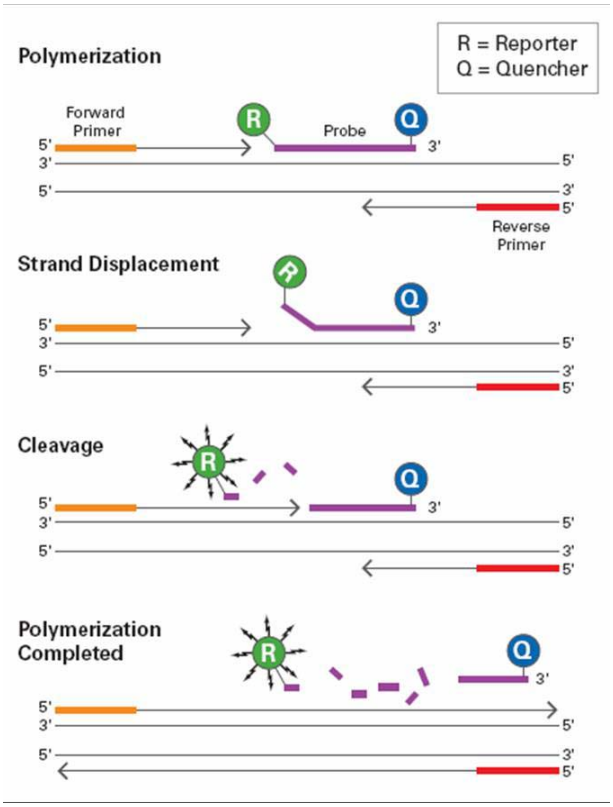

Figure 8.1 Schematic diagram of real time PCR using a fluorescent labelled probe.

8.1 Methods

8.1.1 Comparison of DNA extraction methods

Negative pig faeces were seeded with known amounts of *L. intracellularis* in a serial dilution series from 2.6 x 10^7 to 2.6 x 10^3/ bacteria per 0.2g faeces. *L. intracellularis* DNA was extracted from 0.2g of seeded faeces and 53 known positive samples using two different methods: the Wizard® PCR Preps DNA Purification System (Cat No A7181, Promega, Madison, WI, USA) previously described (Collins and Love, 2007) and the MagMax 96 Viral RNA isolation kit (Cat No AMB 1836, Ambion, Austin, Texas, USA). Faeces contain many PCR inhibitors including bile salts and bilirubin. DNA extracted with the MagMax kit was bound to magnetic beads on a Kingfisher magnetic particle separator and washed away from the PCR inhibitors providing a better quality sample for RT PCR. DNA extracts from both methods were subjected to real time PCR to determine the end point sensitivity and linear range for both extraction methods. A regression plot (Cycle threshold, C_T, versus the number of seeded *L.intracellularis*) was used to determine if the real time PCR was quantitative for the detection of *L.intracellularis* from faecal samples. The mean number of *L. intracellularis* detected was determined for each of the standards on five separate occasions to assess the reproducibility of the Ubi E real time PCR assay. The mean C_T and the standard deviation for the five replicates were analysed.

8.1.2 Development and validation of RT PCR methods

Primer and probe sequences for the real time PCR targeted either the 16S ribosomal RNA gene or the ubiquitone methylase gene (Ubi E) of *L. intracellularis* (Nathues *et al*., 2009). The real time PCR reaction contained 1x RT-PCR buffer (Agpath-ID™ One-Step RT-PCR Kit Cat #AM1005), 12.5 pmol of each primer (Biosearch Technologies, Novato, CA) and 5 pmol of a TaqMan labeled probe (FAM and BHQ-1 quencher), 1µL of 25x RT-PCR enzyme mix, 0.5µL Nuclease-free water and 5µL of DNA template. The amplification protocol consisted of an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C

and 45 sec at 60°C. Reactions were carried out in 96 well plates in an Applied Biosystems 7500 Real-Time PCR thermocycler. The threshold value for each assay was set in the linear range of the sigmoidal amplification plots (Figure 8.2), and the C_T values were determined for each standard or sample by reading the cycle number (from the x axis) where the threshold intersected with the amplification curve.

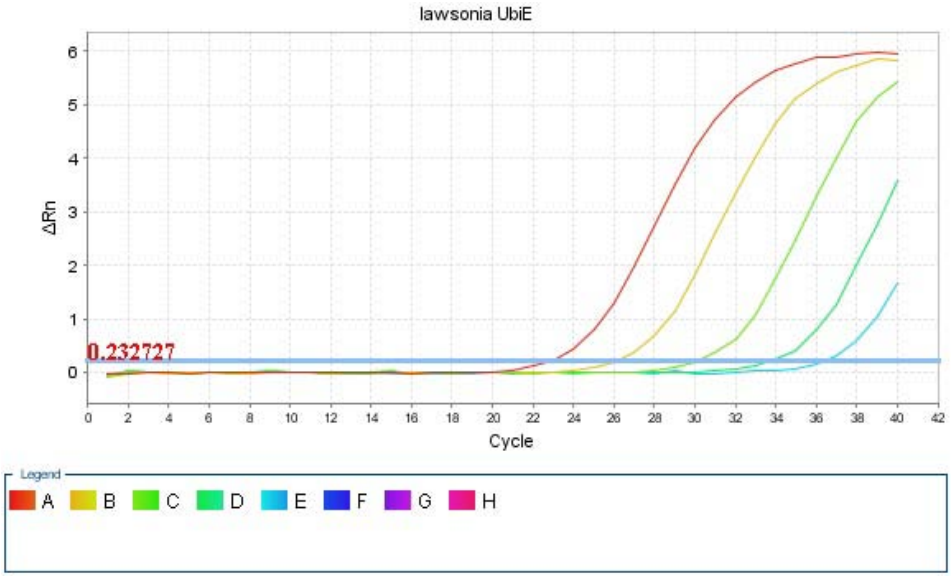

Figure 8.2 - Amplification plots from the *L.intracellularis* Ubi E real time PCR showing the threshold level (0.232727) and cycle threshold (C_T) for each sample or standard, ie. C_T values in these 5 amplification plots are 23.2, 26.3, 30.2, 33.8 and 36.6

The specificity of both real time PCRs was evaluated with DNA (100pg) from other pig enteric bacteria and 3 different isolates of *L. intracellularis* DNA extracted and purified from PHE-affected mucosa (Collins and Love, 2007). DNA extracts of the following bacteria were tested *Brachyspira hyodysentariae*, *B. pilosicoli*, *Escherichia coli* 0141(K85), *E. coli* 0149 (K91, K88), *E. coli* 0157 (K88), *Salmonella choleraesuis*, *Campylobacter mucosalis*, *C. hyointestinalis* and *Desulfovibrio desulfuricans.*

Once optimal DNA extraction and RT PCR conditions were determined, the specificity of the Ubi E real time PCR was further evaluated with a panel of 98 known positive and 100 known negative faecal extracts confirmed with conventional PCR, histological lesions of PE, antibody titre and clinical signs of PE. The proportion of positive pigs with clinical and sub-clinical disease was 18% and 82% respectively. McNemar's chi square test was used to analyse the association between the conventional and real time PCR results.

8.1.3 Evaluation of the Lawsonia qPCR with field samples

Five scouring and five normal faecal samples were collected from 10 commercial herds and tested by qPCR to determine the number of *L.intracellularis* shed per gram of faeces. In addition, up to 40 individual or pooled faecal samples were tested by qPCR from each of five of these herds where we had supporting *L.intracellularis* serology data.

8.1.4. Correlation between the number of *L.intracellularis* shed and other measures of ileitis severity.

Faecal samples previously collected from pigs experimentally infected with about 10^9 *L.intracellularis* (Table 8.1) were tested with the qPCR, and an R square test was used to correlate the number of *L.intracellularis* per gram of faeces with other parameters of disease severity including antibody titres, faecal consistency scores, average daily gain and % area of histological lesions of PE.

Table 8.1 Summary of experimental *L.intracellularis* infection trials

Study	Age of pigs	# pigs	% clinically affected	% sub-clinically affected
1	weaners	60	13%	87%
2	growers	18	33%	66%
3	growers	18	20%	80%

8.2 Results

Comparison of DNA extraction methods

The *L.intracellularis* RT PCR (Ubi E) produced similar C_T values for seeded faeces extracted with either the Wizard or MagMax system, making it difficult to evaluate which extraction method was more sensitive (Table 8.2). Likewise, the RT PCR was able to detect *L.intracellularis* in all 53 known positive faeces (extracted by either the Wizard or MagMax system). The McNemar's Chi square test showed a strong correlation between the Wizard and MagMax extractions when subjected to the *L.intracellularis* RT PCR.

The MagMax extraction system had a distinct time advantage over the Wizard extraction system, which would ultimately lead to reduced test costs. The MagMax system uses robotic technology to extract DNA from 96 faecal samples simultaneously. This is the same format for the automated qPCR, which significantly reduces the assay time (4 fold), compared with the conventional PCR and the Wizard extraction system.

Table 8.2 Comparison of faecal extraction techniques on critical threshold values for RT PCR

Faecal standard	Wizard extraction	Magmax extraction
10^8 L. intracellularis /g	24.8	24.1
10^7 L. intracellularis /g	28.08	27.48
10^6 L. intracellularis /g	31.25	30.77
10^5 L. intracellularis /g	34.12	34.45
10^4 L. intracellularis /g	37.49	38.05

8.2.2 Development and validation of RT PCR methods

The sensitivity of the 16S rRNA PCR appeared to be more sensitive than the Ubi E PCR (5pg DNA) with a decrease in C_T value of 3.3 for every ten-fold dilution of DNA in the quantitative range. However, the specificity of the 16S rRNA RT PCR was poor with water samples giving positive results (Table 8.3).

Table 8.3 - C_T value for DNA dilutions using Ubi PCR and 16S rRNA PCR

DNA concentration	C_T Ubi E	C_T 16S rRNA
5,000pg	30.09	27.44
500pg	33.6	30.86
50pg	37.52	34.12
5pg	38.32	37.85
500fg	Negative	38.71
50fg	Negative	39.56
Water	Negative	38.45

Table 8.4 - C_T values for DNA from other enteric pig pathogens or related bacterial species

Species (100pg DNA)	C_T Ubi E	C_T 16S rRNA
L.intracellularis pig 92	29.49	27.19
L.intracellularis pig 14	38.49	34.71

L.intracellularis pig 13	38.08	31.12
B.hyodysenteriae	Negative	38.47
B.pilosicoli	Negative	36.61
E.coli 0141(k85)	Negative	36.85
E.coli 0149 (K91, K88)	Negative	36.68
E.coli 0157(k88)	Negative	36.66
S.choleraesuis	Negative	36.52
Campylobacter mucosalis	Negative	35.82
C.hyointestinalis	Negative	34.5
Desulfovibrio desulfuricans	Negative	26.42

All 3 different isolates of *L. intracellularis* DNA extracted from pig intestinal samples gave positive results with both real time PCRs. Only the Ubi E PCR was specific for *L.intracellularis*; the 16S rRNA PCR primers and probe amplified DNA from other bacterial species, and were therefore not specific for *L.intracellularis* (Table 8.4)

The specificity of the Ubi E RT PCR on DNA extracted from pig faeces was examined by comparing results from samples previously confirmed as either positive or negative by conventional PCR. Of the 98 known positive samples examined, 97 tested positive by real time PCR. Of the 100 known negative samples, 97 tested negative by real time PCR. Significant association and agreement was demonstrated between the two PCR assays (conventional PCR and new *L.intracellularis* Ubi E RT PCR), using the Fisher's exact chi-square test (contingency table, 2 tail test, $P<0.0001$), the kappa test (significant agreement >0.8) and McNemar's chi square test (significant agreement if value < 3.84) (Table 8.5). The specificity of the Ubi E real time PCR reaction was calculated to be 97% and the sensitivity was 99%.The positive and negative predictive values of the RT PCR compared with the conventional PCR were determined using the following formulas where TP = true positives, FP = false positives, TN = true negatives and FN = false negatives:

Positive predictive value = TP/(TP + FP)
Negative predictive value = TN/(TN+FN)
Sensitivity = TP/(TP+FN)
Specificity = TN/(TN+FP)

Table 8.5 - Summary of significance of agreement between the conventional PCR and *L.intracellularis* Ubi E RT PCR

Statistical test	Significance
Fisher's exact chi-square	<0.0001
Kappa test	0.96
Proportion agreement	9.88
McNemar's chi square	0.25
Probability (chi square)	0.62
Positive predictive value	97%
Negative predictive value	99%
Sensitivity	99%
Specificity	97%

The detection limits of the real-time PCR were between 10^4 and 10^8 *L.intracellularis* per gram of faeces, demonstrated by significant differences ($P<0.0001$) between all standards seeded with 10^4, 10^5, 10^6, 10^7 and 10^8 *L. intracellularis* per gram of faeces (Student's t test). The number of *L.intracellularis* per gram of faeces for unknown samples was determined with the regression equation of C_T value versus the known number of *L. intracellularis* seeded in faecal standards. Quantitative results were only guaranteed if the standards produced a linear regression with an R square >0.98 (Figure 8.3). The reproducibility of *L. intracellularis* quantification by PCR was determined by calculating

the mean, standard deviation and standard error of the mean for the standards in 5 separate RT PCR runs (Table 8.6).

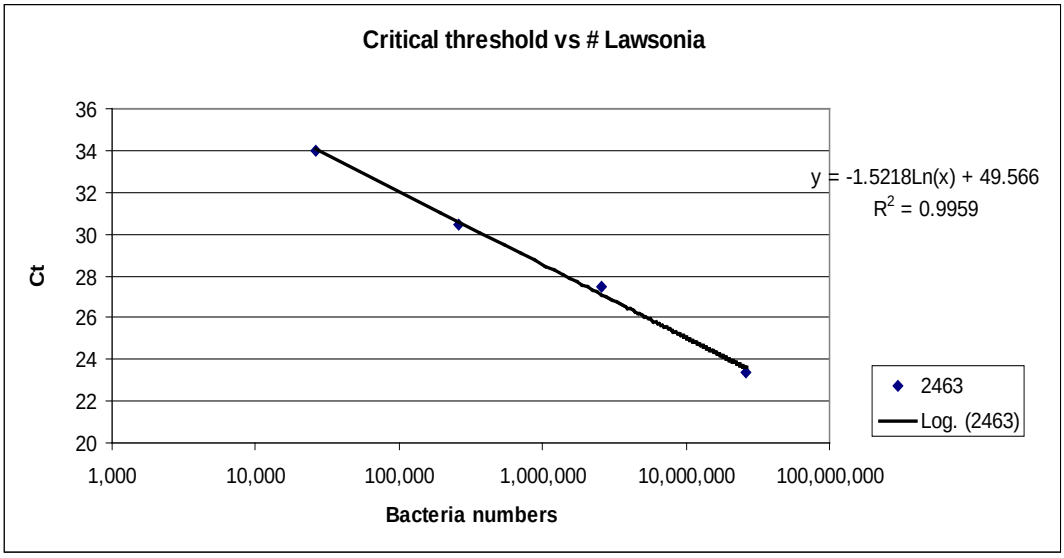

Figure 8.3 - Plot of cycle threshold (C_T) from the real time PCR versus the number of *L.intracellularis* in seeded faecal standards

Table 8.6 - Mean number of *L.intracellularis* (LI) detected (and SEM) per gram of faeces in seeded faecal standards using the Lawsonia Ubi E qRT PCR.

Faecal standards (# LI seeded/g faeces)	# replicates	Mean # LI	Standard Deviation	SEM
1.3×10^8	5	1.45×10^8	1.45×10^7	6.5×10^6
1.3×10^7	5	1.2×10^7	1.7×10^6	7.2×10^5
1.3×10^6	5	1.25×10^6	1.8×10^5	7.3×10^4
1.3×10^5	5	1.4×10^5	1.6×10^4	6.6×10^3
1.3×10^4	5	1.3×10^4	1.8×10^3	1.0×10^3

8.2.3 Evaluation of the *L.intracellularis* qPCR with field samples

Approximately 160 faecal samples were submitted from healthy and scouring pigs on commercial pig farms for testing with the *L.intracellularis* qPCR. The number of *L.intracellularis* detected per gram of faeces was less than 10^6 for the majority of samples (88%), but a small proportion of pigs (1.8%) contained more than 10^8 *L.intracellularis* per gram of faeces (Figure 8.4). Although the diarrhoea score did not correlate well with the number of *L.intracellularis* detected in faeces (r = 0.248), scouring pigs shed significantly higher numbers of *L.intracellularis* than non-scouring pigs (P= 0.041) (Table 8.7).

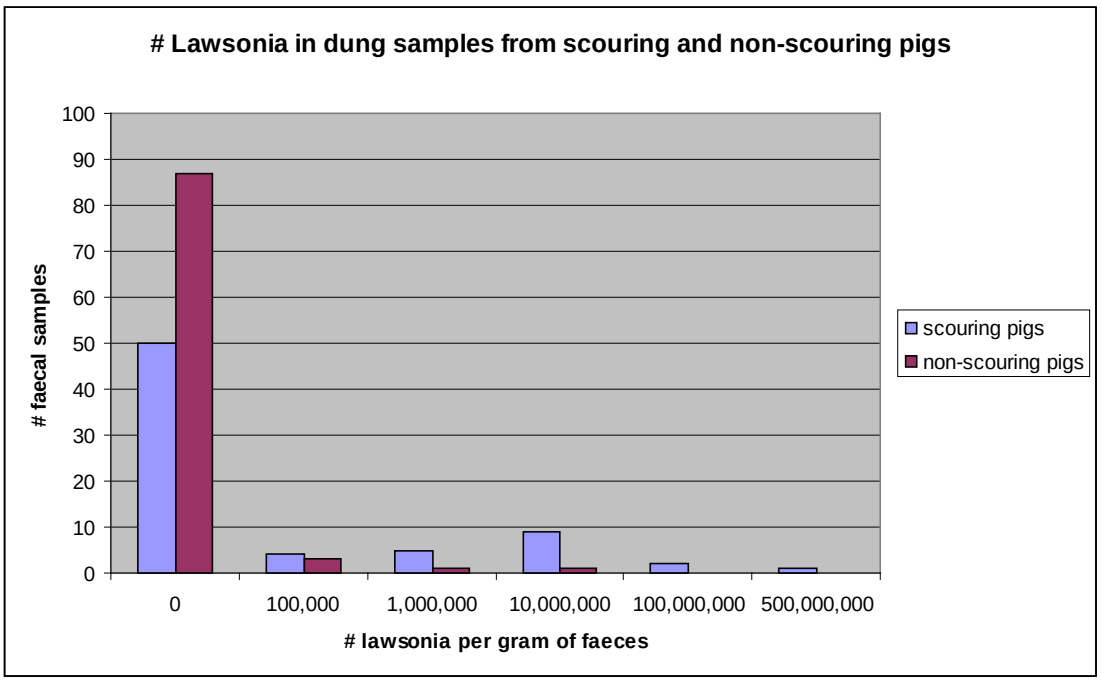

Figure 8.4 - Proportion of scouring and non-scouring pigs shedding sub-clinical (< 10^6) and clinical (>10^6) numbers of *L.intracellularis* per gram of faeces.

Table 8.7 - Mean number of *L.intracellularis* per gram of faeces detected by qPCR in scouring and non-scouring pigs from 10 commercial herds

Health status	Mean # L.intracellularis detected
Non-scouring	$1.04 \times 10^{5\,a}$
Scouring	$3.5 \times 10^{6\,b}$

[ab] Values with different superscripts in the same column indicate significant differences (P<0.05)

Correlation between the number of *L.intracellularis* shed and other measures of ileitis severity.

In order to further validate the quantitative PCR, we have examined correlations between the number of Lawsonia in faeces (determined by qPCR) and other measures of infection and ileitis disease (Table 8.8). In pigs experimentally challenged with *L.intracellularis*, the number of *L.intracellularis* shed at 14 days post challenge correlated strongly (r = 0.68) with the occurrence of diarrhoea between 0 and 21 days post challenge, and may indicate that the qPCR could be used as an early indicator of disease outcomes. A more significant correlation (r = 0.81) was observed between diarrhoea score and the number of *L.intracellularis* at 17 days post challenge. The number of Lawsonia shed at 21 days post challenge correlates well (r = 0.72) with the proportion of the ileum showing histologic lesions of PE at the same time (% area with proliferative lesions and % area with intracellular *L.intracellularis*). In earlier faecal samples from the same animals, moderate correlations exist between *L.intracellularis* numbers at 14 and 17 days post challenge and later histologic lesions (r = 0.43 and 0.51 respectively). Both of these results indicate that the qPCR results will correlate well with ileitis severity in individual pigs.

Table 8.8 - Correlations (r value) between the numbers of *L.intracellularis* (natural log transformed) shed in faeces (qPCR) and other measures of ileitis severity

	Ln # Lawsonia		
Parameter	**14d**	**17d**	**21d**
Scouring 0-21days	0.68	0.26	0.19
Diarrhoea score 17 days	0.66	0.81	0.31

Histopathology 21days	0.43	0.51	0.72
ADG, 0-21 days	-0.39	-0.44	-0.28
ELISA titre 21 days	0.52	0.61	0.50
ELISA titre 28 days	0.33	0.69	0.42

The negative correlations between ADG and the number of *L.intracellularis* in faeces are to be expected, with increasing bacterial numbers leading to reduced pig growth. The magnitude of this correlation is limited by the impact of other factors on pig ADG including genetics, gender and the presence of other diseases. However, we plotted qPCR results with ADG for individual pigs experimentally challenged with *L.intracellularis* to try to identify a critical threshold of *L.intracellularis* where ADG started to decline (Figure 8.5). Pigs shedding between 10^6 and 10^7 *L.intracellularis* per gram of faeces showed reduced ADG in the three weeks post *L.intracellularis* challenge. Generating similar data from commercial herds would provide a more meaningful threshold for producers to be able to quantify the cost of sub-clinical ileitis in their herds and the financial benefit of control measures.

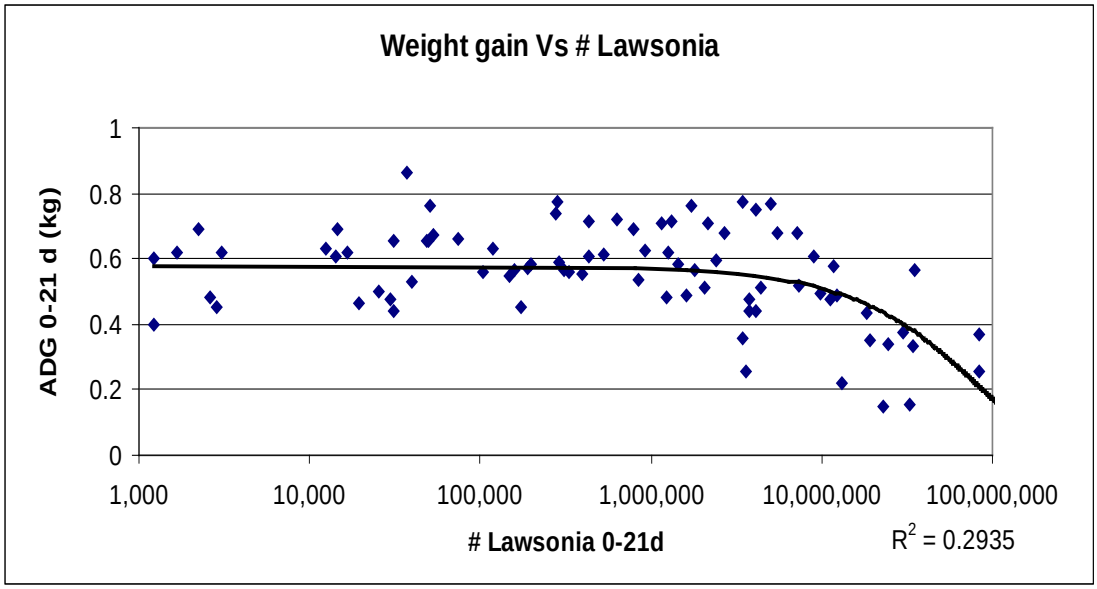

Figure 8.5 - Relationship between average daily gain and the number of *L.intracellularis* detected in faeces for grower pigs experimentally challenged with *L.intracellularis*.

Pilot study to assess the ability of the *L.intracellularis* qRT PCR to monitor ileitis control in pigs experimentally challenged with *L.intracellularis*

The qPCR has also been able to show the effect of antibiotics on reducing the faecal shedding of *L.intracellularis* in pigs experimentally challenged with *L.intracellularis*. Pigs medicated continuously with low levels of tylosin and oxytetracycline had significantly fewer *L.intracellularis* shed in their faeces ($P<0.05$) from 14 days post infection onwards (Table 8.9). This was associated with reduced clinical signs in medicated pigs compared with unmedicated controls.

Table 8.9 - Natural log of the mean number of *L.intracellularis* per gram of faeces detected by qPCR in pigs medicated with antibiotics prior to challenge with virulent *L.intracellularis*.

	Mean Ln *L.intracellularis* shed per gram of faeces				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Non-medicated	0	0.08^a	3.35^a	4.05^a	1.45^a
100ppm OTC	0	0.31^a	0.51^b	0.50^b	0.04^b
50ppm OTC	0	0.25^a	2.76^a	1.41^b	0.71^a
50ppm Tylan	0	0^a	0.56^b	0.16^b	0.20^b

[ab] Values with different superscripts in the same column indicate significant differences (P<0.05)

In the second study, medication of *L.intracellularis* infected pigs with CTC or OTC at 400ppm or Tiamulin at 200ppm two weeks after experimental infection prevented diarrhoea and reduced weight gains relative to the non-medicated pigs. The qPCR was able to demonstrate that the number of *L.intracellularis* decreased significantly within 6 days of chlortetracycline or tiamulin treatment in pigs with ileitis (Table 8.10).

Table 8.10 - Mean number of *L.intracellularis* (Ln) detected in pigs experimentally challenged with *L.intracellularis* and medicated from 14 days post challenge with 400 ppm oxytetracycline (OTC), 400ppm chlortetracycline (CTC), 200ppm Tiamulin or non-medicated.

	Ln # L.intracellularis			
Group	**14 days**	**20 days**	**24 days**	**28 days**
Non-medicated	5.18 [a]	4.75 [a]	3.19 [a]	1.85 [a]
400ppm OTC	3.81 [a]	5.05 [a]	2.04 [a]	2.04 [a]
400ppm CTC	2.64 [a]	0 [b]	0 [b]	0 [b]
200ppm Tiamulin	4.18 [a]	1.14 [c]	0.23 [ab]	0.28 [ab]

[ab]Values in same column with different superscripts indicate significant differences (P<0.05)

The qPCR was also able to show reduced numbers of *L.intracellularis* in pigs vaccinated with Enterisol Ileitis relative to non-vaccinated pigs, following experimental challenge (Table 8.11). The qPCR therefore looks capable of monitoring control of ileitis, either by vaccination or antibiotic medication in individual pigs.

Table 8.11 - Mean number of *L.intracellularis* (LI) detected by qPCR in weaners vaccinated with Enterisol Ileitis (or non-vaccinated) and experimentally challenged with *L.intracellularis* 6 weeks later

	# LI @ 14 days	# LI @ 17 days	# LI @ 21 days
Vaccinated + LI challenge	4.5×10^{6a}	5.8×10^{5a}	1.1×10^{6a}
Non-vaccinated + LI challenge	7.1×10^{6b}	1.5×10^{6b}	2.2×10^{6a}

[ab]Values in same column with different superscripts indicate significant differences (P<0.05)

Monitoring *L.intracellularis* in rats using the qPCR

The *L.intracellularis* qPCR was able to detect *L.intracellularis* in the intestines and faeces of rats trapped on pig farms. In some herds, as many as 83% of rats shed Lawsonia in their faeces, with most rats shedding less than 10^5 Lawsonia per gram of faeces. However a small proportion of rats shed a staggering 10^9 Lawsonia per gram of faeces (Table 8.12).

Table 8.12 - Total percentage of rats shedding *Lawsonia intracellularis* and the percentage shedding at high (more than 1×10^7), medium (10^5-10^7) or low (10^3-10^5) numbers of *L.intracellularis*/g faeces, or undetectable numbers in each piggery.

Farm	Number of rats tested	Total % PCR positive rats	Percentage of rats shedding *L.intracellularis* at a range of concentrations			
			> $1x10^7$/g faeces	10^5-10^7/g faeces	10^3-10^5/g faeces	Undetected
A	28	3.6%	0%	0%	3.6%	96.4%
B	119	70.6%	4.7%	13.4%	52.5%	29.4%
C	60	83.4%	10%	30%	43.4%	16.6%

D	120	4.4%	0%	0%	4.4%	95.6%

8. Discussion

Rabbit anti-*L.intracellularis* serum (R97) produced in this project is as sensitive as the commercially available monoclonal antibodies to *L.intracellularis* (IG4 and VPM53). The polyclonal R97 rabbit serum reacts primarily to the same *L.intracellularis* protein as the two monoclonal antibodies in a wide range of *L.intracellularis* isolates, and does not cross-react with other common pig enteric pig pathogens. However, it appears the R97 rabbit sera cross-reacts with other proteins found in the faeces of pigs, and adsorbing the R97 sera with healthy pig enterocytes did not remove this cross-reacting antibodies. We are yet to test whether affinity purification of the R97 sera will improve the specificity of the LFIA. It is also possible that a monoclonal antibody to *L.intracellularis* (produced in mice) would not react with negative pig faeces, and this would be worth investigating with a commercial partner.

The *L.intracellularis* quantitative PCR has a significant advantage over the conventional PCR in being able to reliably quantify the number of *L.intracellularis* shed in the faeces of pigs clinically and sub-clinically affected with ileitis. The qRT PCR was developed to validate the results of the semi-quantitative faecal immunoassay for ileitis. However, the reproducibility of results and the strong correlations between the qRT PCR and other measures of ileitis severity indicate that the qRT PCR could provide producers with a new tool to monitor the severity of *L.intracellularis* infection in their herd and evaluate current management strategies (vaccination, antibiotics and improved hygiene).

Although the qRT PCR is already able to quantify *L.intracellularis* shed in the faeces of experimentally infected pigs, some additional work is required to optimise the assay for use at the pig herd level, where it will be economically viable. Specifically, optimal sample sizes for pooled faeces will need to be determined to detect herds clinically and sub-clinically affected with ileitis, and qRT PCR results will need to be correlated with *L.intracellularis* serology and growth records in the herds.

9. Budget Summary

Funding allocation	2008-2009	2009-2010	Total
Salary	$49,630.92	$91,279.08*	$140,910
Operating	$49,849.08	$8,120.92	$57,970
Total Pork CRC cash allocated:	$99,480	$99,400	$198,880

*A transfer of $33,340 from the operating to salaries budget on 30/9/10 was reversed as it was ultimately not required due to the provision of additional TO support from NSW DPI.

In-kind contributions Per Year	2008-2009	2009-2010	2010-2011	Total
Research Organisation Category of in-kind staff	0.66	1.1	1.1	2.86
Total other non-staff in-kind contributions ($)	$42,800	$55,100	$47,800	$145,700

10. Acknowledgements

I would like to thank and acknowledge the invaluable support of staff at Elizabeth Macarthur Agricultural Institute, in particular Shayne Fell and Paul Young. The producers and consulting veterinarians who provided samples and production information were also an integral part of this project. Statistical support was provided by Idris Barchia from Elizabeth Macarthur Agricultural Institute. Finally, I would like thank the Pork CRC for

their financial support and the opportunities to present research outcomes to producers and Ross Cutler in particular for his sane advice when obstacles had to be overcome.

11. References

Bangs Laboratories, 2008. Working with microspheres. Tech note 201.

Bangs Laboratories, 2008. Washing microspheres. Tech note 203.

Bangs Laboratories, 2008. Adsorption to microspheres. Tech note 204.

Bangs Laboratories, 2008. Lateral flow tests. Tech note 303.

Bangs Laboratories, 2009. Microsphere aggregation. Tech note 202.

Brüning, A., Bellamy, K., Talbot, D. and Anderson, J. 1999. A rapid chromatographic strip test for the pen-side diagnosis of rinderpest virus. *J. Virolog. Methods*, 81: 143-154.

Collins, A.M. and Love, R.J., 2007. Re-challenge of pigs following recovery from proliferative enteropathy. Vet. Micro. 120, 381-386.

Collins, A.M., Fell, S., van Straaten, J. and Bolsius, N. Reduced muscle growth in pigs sub-clinically affected with proliferative enteropathy. *Proceedings of the 21st International Pig Veterinary Congress*, Vancouver, Canada, 18-21 July, 2010a:234.

Collins, A.M., Fell, S., van Straaten, J., Bolsius, N., Penrose, L. Increased variation in pig weight and reduced feed intake in pigs sub-clinically affected with proliferative enteropathy. *Proceedings of the 21st International Pig Veterinary Congress*, Vancouver, Canada, 18-21 July, 2010b:690.

Guardian Biosciences. http://guardianbiosciences.com

Henderson, K and Stewart, J. 2000. A dipstick immunoassay to rapidly measure serum oestrone sulphate concentrations in horses. *Reprod. Fertil. Dev.*, 12:183-189.

Holyoake, P.K., Collins, A., and Mullan, B. Simulation of the economic impact of *Lawsonia intracellularis* infection. *Proceedings of the 21st International Pig Veterinary Congress*, Vancouver, Canada, 18-21 July, 2010a:223.

Holyoake, P.K., Emery, D., Gonsalves, J., Donahoo, M. and Collins, A. 2010b. Prevalence of antibodies to *Lawsonia intracellularis* in pig herds in Australia. *Aust. Vet.J.*, 88: 186-188.

McOrist, S., Boid, R., Lawson, G.H.K. and McConnell, I. 1987. Monoclonal antibodies to intracellular campyocater-like organisms of the porcine proliferative enteropathies. *Vet. Rec.*, 121: 421-422.

Millipore Corporation, 2002. Rapid lateral flow test strips: considerations for product development.

Reid, S.M., Ferris, N.P., Brüning, A., Hutchings, G.H., Kowalska, Z. and Akerblom, L. 2001. Development of a rapid chromatographic strip test for the pen-side detection of foot-and-mouth disease virus antigen. *J. Virolog. Methods*, 96: 189-202.

Tan, J. 2008. Analysis of whole blood samples: optimization of sample preparation for rapid analysis. Master of Engineering thesis, Cornell University, USA.

Warwick, C.J. 2008. An assessment of the suitability of using streptavidin coated microspheres on the test line of a lateral flow immunoassay with a hydrophilic membrane. Master of Science thesis, Cranfield University, UK.

12. Publications and Presentations

Collins, A.M., Fell, S., Pearson, H. and Toribio, J.-A. 2011. Colonisation and shedding of *Lawsonia intracellularis* in experimentally inoculated rodents and in wild rodents on pig farms. Vet. Microbiol. 150: 384-388.

Collins, A. Getting the most out of new and existing diagnostics for the control of proliferative enteropathy. *Proceedings of Aust. Pig Vets.*, Melbourne 2011.
Collins, A. Reducing the impact of ileitis in your herd. *Bendigo Pig Fair* 2011.
Collins, A. Controlling the impact of ileitis in your herd. *CHM Alliance workshop* 2010.

Appendix A: Investigation of immunoassay platforms for the detection of *L. intracellularis* in faecal samples

Prior to investigating platforms for the faecal immunoassay (dipstick or magnetic bead capture), I became aware of an Elanco test for the detection of *L.intracellularis* in porcine faeces, called 'Lawsonia FIRST'. I met with Elanco's Business unit Manager for intensive livestock (Alex Turney) to discuss the validation, application and marketing of their test, in order to determine whether it was worth continuing with the development of the Pork CRC funded faecal immunoassay for *L.intracellularis*. The Lawsonia FIRST test appeared to have limitations with respect to field use, validation and applications as outlined below, and this guided our continued research.

Ease of application

The Lawsonia FIRST test was designed as a field test, but requires an Elanco operator to perform the test on farm. The test uses magnetic beads coated with *L.intracellularis* antibodies to capture *L.intracellularis* antigen in faeces. The test must be transported carefully by the Elanco field staff as the antibody can easily be unbound from the beads, reducing the accuracy of the test. In addition, the test requires a magnetic tube holder and currently Elanco only have one magnet tube holder in Australia (due to the cost), limiting the accessibility of the test.

Sampling protocol

Elanco recommend testing 10 individual faecal samples from pigs between 12-15 weeks of age to demonstrate the presence of *L.intracellularis* in the herd. The results of samples collected at this time correlate well with faecal PCR and serology. However, faecal PCR and serology have a distinct advantage over the Elanco test as they are able to diagnose ileitis in younger pigs, thereby giving the producer an earlier warning to avoid production losses due to ileitis.

Sensitivity and specificity

The Lawsonia FIRST test is a qualitative, not quantitative test. It can only be used to demonstrate the presence or absence of *L.intracellularis* in pig herds. As previous serological studies in Australia have shown that 100% of pig herds are infected with *L.intracellularis*, its usefulness as a herd diagnostic assay in Australia is limited. The antibody used in the Elanco test reacted with 8 different *L.intracellularis* isolates in US studies (Connie Gebhart at the University of Minnesota). Elanco have not evaluated the test with Australian isolates of *L.intracellularis*, but variability between isolates of *L.intracellularis* is minimal. The Elanco antibody does cross-react with *Staphylococcus aureus*, so an *S.aureus* antibody has been added to the kit to avoid this cross reaction.

Applications in Australia

Elanco are not charging for the test as they are using it as a marketing tool to be invited onto farms to discuss whole herd health and production management. Elanco have stated that the test will be used as a marketing tool for their products, not limited to the sale of Tylan for the control of ileitis. They see their role in advising producers on the cumulative benefits of control of weaner diseases with Pulmotil, control of ileitis in growers with Tylan and improved finisher growth with Paylean. Elanco believe that the test is more applicable in smaller Asian pig herds where the confirmed diagnosis of ileitis is less common, due to poor accessibility to laboratory and veterinary support.

The Elanco test is not able to evaluate current ileitis control strategies on farm or indicate the level of disease in the herd. In contrast the assays we are developing are aimed at evaluating the efficacy of antibiotic medication, vaccination or improved hygiene to control ileitis, which requires a semi-quantitative assay. We intend to validate the sensitivity of the faecal immunoassay with a quantitative PCR.

I contacted a number of pig veterinary consultants in Australia who had attended seminars on the Elanco Lawsonia FIRST test. All have said that they still saw the need for a semi-quantitative real-time diagnostic assay for ileitis (Pork CRC project 2C-109), specifically to evaluate their ileitis control strategies. One vet said "I imagine we would use the test regularly on selected sites and ages for each quarter of the year, and occasionally at other times to assist in decision making, not just for medication, but also hygiene and other management factors."

We decided to continue to develop a faecal immunoassay for *L.intracellularis*, but focussed on the lateral flow immunoassay platform to overcome the problems that Elanco are experiencing with their antibody coated magnetic beads. We also decided to develop a quantitative PCR to validate the sensitivity of the faecal immunoassay.