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Immunity to *Lawsonia intracellularis*

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The porcine proliferative enteropathies (PPE) are a group of conditions that affect pigs of all ages housed in a range of management systems worldwide. The chronic form of PPE, commonly known as 'ileitis,' may lead to clinical signs including diarrhea and reduced weight gain. Proliferative hemorrhagic enteropathy (PHE), the acute form of disease, is characterized by bloody diarrhea caused by a massive hemorrhage from the ileal mucosa. The etiologic agent of the porcine proliferative enteropathies is *Lawsonia intracellularis*, an obligate intracellular bacterium.

PPE is currently controlled by medicating pigs in-feed or in-water using a range of antibiotics[1,2,3,4,5]. Some current medication strategies recommend preventing *L. intracellularis* infection by continuous medication with high levels of antibiotics, which may simply extend the period of susceptibility to *L.intracellularis*, delaying infection until the removal of antibiotics. This becomes a problem when susceptible finisher and breeding pigs become infected with *L.intracellularis*. Finisher pigs infected with *L.intracellularis* experience more severe clinical signs of disease and a greater mortality than younger pigs[6].

The suggestion that pigs could develop immunity to re-infection with *L.intracellularis* came from the observation of two sequential outbreaks of PHE in a minimal disease pig farm[7]. The first outbreak affected highly susceptible breeding sows and boars of all ages, but was controlled with the addition of antibiotics to the feed. Seven weeks later the second outbreak occurred in gilts and young boars introduced into the breeding unit just before, during or after the in-feed medication was administered. It is probable that antibiotics in the feed of young pigs prevented infection and the development of immunity, and maintained susceptibility to *L.intracellularis* until these pigs were moved into the breeding unit and off feed containing medication.

Strategically medicating pigs with antibiotics in combination with exposure to *L.intracellularis* could permit the development of immunity to *L.intracellularis* while avoiding clinical signs of disease. One possible strategy would involve treating *L.intracellularis*-infected pigs with high levels of antibiotics in-feed once clinical signs are observed. A modification of this strategy successfully controlled PHE in newly introduced breeding animals. Pigs were exposed to infection for 21 days, then treated with antibiotics, before mixing the new pigs into the herd[8].

An alternative strategy to control PPE (ileitis) recommends continuous medication of diets with low levels of antibiotics. When the pigs are exposed to *L.intracellularis*, the antibiotics may subdue disease and allow subclinical infection and the development of immunity to re-infection[9].

In this study we investigated whether pigs previously infected with *L.intracellularis* were immune to *L.intracellularis* on further exposure. The study also aimed to identify antibiotic medication strategies that allowed the development of immunity to *L.intracellularis*, but avoided clinical signs of disease.

In order to develop strategic medication protocols to induce immunity to *L.intracellularis* in commercial pig farms, we also needed to investigate the effect of varying the dose of bacteria on immunity to *L.intracellularis*. It is probable that the ingestion of infectious feces by susceptible pigs would deliver lower doses of *L. intracellularis* compared with our experimental inoculations. The aim of the last experiment was to determine the initial minimum dose of *L. intracellularis* necessary to induce immunity to a higher dose of *L. intracellularis* on re-inoculation.

Demonstration of *L.intracellularis* infection in weaned pigs

A reliable model for the experimental infection of pigs with *L.intracellularis* was developed to enable the investigation of immunity to *L.intracellularis*. Previous experiments suggested that Large White, Landrace cross pigs were susceptible to *L.intracellularis* infection and developed clinical signs of disease. Piglets were weaned at 3–4 weeks of age into individual pens or weaner crates with solid walls and elevated floors to minimise the possible transmission of infection.

L.intracellularis bacteria were extracted from homogenized PHE-affected tissue as described previously[10] and an aliquot of the bacterial suspension was stained by an indirect immunofluorescent assay to enumerate the num-

ber of *L.intracellularis* per milliliter of inoculum. Pigs were orally inoculated with 25mL of bacterial suspension containing between 10^9 and 10^{10} *L.intracellularis* via a tube passed well into the esophagus.

Pigs were monitored for clinical signs of disease including diarrhea and reduced weekly weight gain. Blood samples were collected weekly for the detection of serum IgG antibodies against *L.intracellularis*, by an indirect immunofluorescence test (IFAT)[11]. Fecal samples were collected twice weekly for PCR amplification of *L.intracellularis* DNA[12] and immunofluorescent staining of fecal smears[13]. Feces and blood were collected prior to oral inoculation to verify that pigs were naïve to *L.intracellularis*. In the initial experiments one inoculated and one control pig was euthanased at 21 days post-inoculation (pi), and sections of the ileum and colon were preserved in buffered formalin for hematoxylin and eosin staining.

A temporal pattern of *L.intracellularis* infection was identified using the above infection model. Pigs orally inoculated with between 10^9 and 10^{10} *L.intracellularis* developed clinical signs of ileitis including diarrhea and reduced weight gains between 14 and 21 days pi. Prior to clinical signs becoming apparent, PCR and immunofluorescent staining demonstrated consistent fecal shedding of *L.intracellularis* from 7 days pi onwards. Following the development of clinical signs, histologic lesions of PPE were observed in the terminal ileum of the inoculated pig necropsied at 21 days pi. The development of an immune response to *L.intracellularis* was evident between 3 and 8 weeks pi.

Demonstration of immunity to *L.intracellularis*

The development of immunity to re-infection with *L.intracellularis* was tested by re-inoculating previously infected pigs after the fecal shedding of *L.intracellularis* had ceased to be detected. Re-inoculated pigs were observed for clinical signs of disease, and the feces and serum were monitored for evidence of *L.intracellularis* infection. Pigs previously infected with *L.intracellularis* failed to shed detectable numbers of *L.intracellularis* in their feces and did not develop clinical signs of disease post-secondary inoculation. The absence of *L.intracellularis* in the feces of re-inoculated pigs suggests that *L.intracellularis* were inactivated before entry and colonization of mucosal cells.

The initial immune response to *L.intracellularis* would probably be generated in the mesenteric lymph nodes (MLN), where bacteria that pass through the intestinal epithelium arrive via the lamina propria (LP). B cells in the MLN are differentiated for IgA and IgG production in response to antigenic stimulation. The small number of IgG producing plasma cells are directed to peripheral areas, where their product can be detected by serology. The IgA-producing plasma cells are directed back to the LP, where secreted IgA is translocated through the epithelium and is available to bind to previously experienced antigens in the intestinal lumen. It is probable that secretory IgA specific for *L.intracellularis* was present in the intestinal lumen when the study pigs were re-inoculated. This secretory IgA would prevent the entry of *L.intracellularis* into mucosal cells and reduce the secondary immune response to *L.intracellularis*.

Some pigs did develop a secondary serological response to *L.intracellularis*. These pigs may have been responding to the *L.intracellularis* antigen in the intestinal lumen or may have become infected with a low level of *L.intracellularis*. If re-infection did occur, the number of *L.intracellularis* shed in the feces was lower than the detection sensitivity of the PCR (approximately 4 x 10^2 *L.intracellularis*/gram of feces).

The development of immunity to *L.intracellularis* in pigs medicated with antibiotics

Antibiotic medication was introduced into the development of immunity model described previously to reduce clinical signs of disease. Antibiotics were added either before or after the primary inoculation to modify the *L.intracellularis* infection. Diets containing oxytetracycline at 50ppm, 100ppm, 300ppm, and 600ppm were supplied to groups of pigs four days prior to oral inoculation with *L.intracellularis*. Tylosin at 50ppm and 100ppm, chlortetracycline at 400ppm, and olaquindox at 25ppm, 50ppm, and 100ppm were also supplied in-feed to groups of pigs 4 days prior to inoculation with *L.intracellularis*. Another group of pigs was inoculated with *L.intracellularis* before antibiotic medication with 400ppm of chlortetracycline from 14 to 24 days pi. Separate groups of pigs were maintained on non-medicated feed for all of these experiments to verify the infectivity of each inoculum.

All of the pigs were orally inoculated with a sufficient number of *L.intracellularis* to cause clinical signs of ileitis in unmedicated pigs. Pigs were monitored for clinical signs of disease, evidence of fecal shedding of *L.intracellularis* and a serological immune response against *L.intracellularis* as described in the original infection model.

Infection and the development of immunity were affected in three different ways in pigs continuously medicated with antibiotics prior to inoculation with *L.intracellularis*. Some concentrations of antibiotics prevented *L.intracellularis* infection and disease in all pigs and extended the susceptibility of pigs to *L.intracellularis* until

the antibiotic was removed. Pigs maintained on feed medicated with oxytetracycline at 300 and 600ppm, chlortetracycline at 400ppm, or olaquindox at 100ppm were all totally protected from the first experimental dose of *L.intracellularis*, and did not develop immunity to re-infection with *L.intracellularis*. On re-inoculation, all of these previously uninfected pigs became infected with *L.intracellularis* and developed clinical signs of disease, developing the same temporal pattern of infection as non-medicated pigs.

Pigs maintained on non-medicated feed or pigs medicated with oxytetracycline at 50 or 100ppm, or 50ppm tylosin, were not protected and all became infected with *L.intracellularis*. These pigs shed *L.intracellularis* in their feces and developed a serological response against *L.intracellularis* at similar time points post-inoculation. Pigs medicated with relatively low levels of oxytetracycline or tylosin developed less severe clinical signs of disease than the pigs fed non-medicated diets. All of these previously infected pigs were immune to re-infection with *L.intracellularis*.

The third situation involved groups of pigs that differed in their response to the primary inoculation and therefore also to the development of immunity. Diets containing olaquindox at 25 or 50ppm, or tylosin at 100ppm, did not prevent every pig from becoming infected with *L.intracellularis*. Fecal shedding of *L. intracellularis* and the development of an immune response was delayed for up to 2 weeks in those pigs that did become infected compared with non-medicated pigs. Pigs that had become infected with *L.intracellularis* following primary inoculation were immune to re-infection, regardless of the in-feed medication. Pigs on these medications that had been protected by the above medications post-primary inoculation (i.e., had not shed *L.intracellularis* in their feces) were not immune to *L.intracellularis*, but were susceptible to *L.intracellularis* on secondary inoculation.

The most successful strategies to induce the development of immunity to *L. intracellularis* allowed subclinical infection of pigs that were continuously medicated with low levels of antibiotics. The level of antibiotic medication necessary to allow subclinical infection and immunity may also depend on the level of exposure of pigs to *L. intracellularis*.

Strategic antibiotic medication to induce immunity to *L.intracellularis*

The treatment of pigs with 400ppm of chlortetracycline in-feed from 14 to 24 days pi terminated the fecal shedding of *L.intracellularis* and suppressed the primary serological immune response to *L.intracellularis*. Clinical signs of disease were also abbreviated and reduced in severity in pigs medicated with 400ppm chlortetracycline compared with non-medicated pigs.

The development of immunity to re-infection with *L.intracellularis* was demonstrated following re-exposure, when fecal shedding of *L.intracellularis* was not detected. An anamnestic serological IgG immune response to *L.intracellularis* was detected in most of the pigs. The intensity of the secondary immune response was greatest in the pigs that had not developed a detectable IgG immune response post-primary inoculation.

Implementing this medication strategy to control ileitis has many potential problems with regards to synchronizing the timing of *L.intracellularis* infection and also the introduction of antibiotic medication. If medication is delayed beyond 14 days after exposure, pigs may develop more severe clinical signs of disease. If medication is introduced before the initiation of an immune response to *L.intracellularis*, pigs may not develop immunity to re-infection. Variation in the dose of *L.intracellularis* may also affect the temporal pattern of *L.intracellularis* infection.

The effect of dose on the development of immunity to *L.intracellularis*

It was important to determine if natural exposures to *L.intracellularis* (via infectious feces) were sufficient to induce immunity to re-infection with *L.intracellularis*. It was previously calculated that experimentally infected pigs excrete between 5(10^4 and 7(10^8 *L.intracellularis* per gram of feces[14]. The aim of this experiment was to determine the minimum dose of *L. intracellularis* necessary to induce immunity to a higher dose of *L. intracellularis* on re-inoculation.

Five groups of pigs were inoculated with variable numbers of *L.intracellularis*. Group 1 pigs were not inoculated. Group 2, 3, 4, and 5 pigs were inoculated with 2(10^3, 2(10^5, 2(10^7, and 2(10^{10} *L. intracellularis*, respectively. Pigs were observed daily for clinical signs of disease and infection was monitored by serology and fecal PCR for 10 weeks pi.

At 70 days pi, all Group 2 to 5 pigs and a sixth group of susceptible recently weaned pigs were orally inoculated with a suspension of homogenized PHE-affected mucosa containing approximately 1 x 10^{10} *L. intracellularis*. Clinical signs of disease and infection were monitored as described above.

Severe clinical signs of ileitis (PPE) were observed in the pigs inoculated with 10^{10} *L. intracellularis* (Group 5). Persistent diarrhea between 14 and 26 days pi necessitated the treatment of a number of Group 5 pigs with antibiotics from 24 days pi. Substantial average weekly weight gain reductions were observed in Group 5 pigs between 2 and 4 weeks pi (**Figure 1**). Milder clinical signs

Figure 1. The average weekly weight gain of pigs inoculated with 10^{10} (Group 5), 10^{7} (Group 4), 10^{5} (Group 3), 10^{3} (Group 2) *L.intracellularis*, or uninoculated pigs (Group 1).

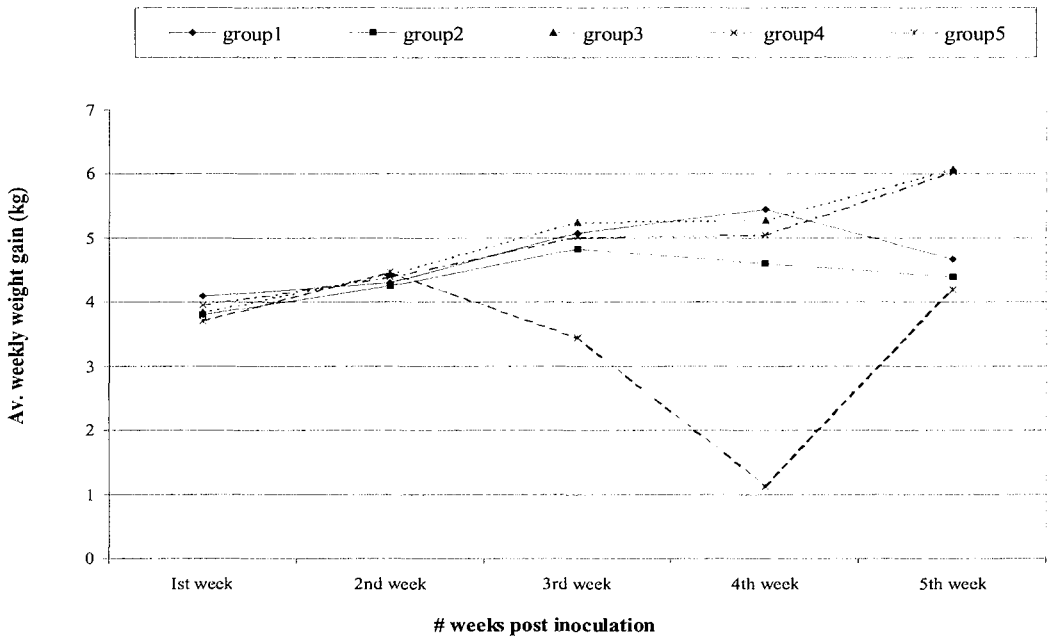

Table 1. Summary of pattern of infection of pigs inoculated with a varying dose of *L. intracellularis*.

Group #	Estimated dose of *L. intracellularis*	Days pi when 80% pigs PCR positive	Days pi when 80% pigs IFAT positive
2	2.0×10^{3}	26–54 days	56–70 days onwards
3	2.0×10^{5}	19–33 days	56–70 days*
4	2.0×10^{7}	14–28 days	35–49 days
5	2.0×10^{10}	7–44 days	21–70 days onwards

*Only 2 of 5 pigs developed a detectable serological response.

of ileitis (PPE) were observed in pigs inoculated with 10^{7} *L. intracellularis* (Group 4), including intermittent diarrhea between 19 and 21 days pi. Diarrhea was not observed in the pigs given lower doses of *L. intracellularis* (Groups 2 or 3) or not inoculated (Group 1). Reduced average weekly weight gains were not observed in any of the Group 4, 3, 2, or 1 pigs.

Fecal shedding of *L.intracellularis* was detected in the pigs given the highest dose of *L.intracellularis* (Group 5) between 7 and 44 days pi. There was a delay in the detection of fecal shedding of *L. intracellularis* in pigs given lower doses of *L. intracellularis*. Fecal shedding was detected in the majority of Group 4, Group 3, and Group 2 pigs from 14, 19, or 26 days pi, respectively (**Table 1**). The earliest serological response to *L. intracellularis* was detected in the highest dose pigs at 21 days pi, and was delayed in pigs given lower doses of *L. intracellularis* (Table 1).

Following re-inoculation of the Group 2 to 5 pigs, fecal shedding of *L.intracellularis* was not detected at any time between 0 and 30 days post-secondary inoculation. The sixth group of pigs, not previously infected with *L.intracellularis*, shed *L.intracellularis* in their feces continuously from 7 days pi onwards, and developed a serum IgG response to *L.intracellularis* from 14 days pi onwards. Serum IgG antibodies against *L.intracellularis* continued to be detected in the previously infected Group 2 to 5 pigs, with some pigs showing a boost in intensity of the IgG immune response after re-inoculation.

It was not possible to determine the minimum dose of *L.intracellularis* necessary to induce immunity because pigs inoculated with the lowest dose of *L.intracellularis* (10^{3} bacteria) demonstrated immunity to re-infection. It was previously calculated that experimentally infected pigs could excrete between $5(10^{4}$ and $7(10^{8}$ *L.intracellularis* per gram of feces[14]. It therefore appears

likely that as little as 0.1g of infected feces will provide sufficient *L.intracellularis* bacteria to induce immunity to re-infection with a much higher number of *L.intracellularis*.

Duration of immunity

None of the above studies examined whether immunity to *L.intracellularis* could extend beyond 7 weeks after the fecal shedding of *L.intracellularis* ceased to be detected. Considering that *L.intracellularis* infection was found to be most prevalent in endemically infected herds between 10 and 24 days after weaning[15], it would be useful to know if the duration of immunity to *L.intracellularis* could be extended for the lifetime of normal market pigs and breeding stock.

Maternal protection against *L.intracellularis* infection

Previous serological studies of natural outbreaks of PPE (ileitis) have suggested that high serum IgG titres against *L.intracellularis* detected in some 3-week-old pigs indicated maternally acquired antibody[16]. The importance of this maternally derived antibody in protecting piglets against *L.intracellularis* infection has yet to be determined. Piglets may also be protected from *L.intracellularis* infection via the ingestion of sow's milk containing IgA antibodies against *L.intracellularis*, which may delay the piglets' susceptibility to *L.intracellularis* infection until weaning.

We investigated if sows can provide protection against *L.intracellularis* infection to their piglets via milk, and whether such protected piglets were susceptible to a later *L.intracellularis* challenge.

Three sows were obtained from two large commercial pig farms with differing *L.intracellularis* infection health statuses. Sows 1 and 2 came from a herd considered to be endemically infected with *L.intracellularis*[7]. Sow 3 arrived mid-gestation from a herd with no previous history of PPE (ileitis). Three piglets from each sow were weaned at approximately 21 days and a further three piglets from sows 1 and 2 and eight piglets from sow 3 remained on the sow for an additional 2 weeks.

The three weaned and three unweaned piglets from each sow were orally inoculated with a suspension of homogenized PHE-affected mucosa containing approximately 9(10^9 *L.intracellularis*. Five unweaned pigs from sow 3 were not inoculated with *L.intracellularis*, but shared the farrowing crate with their inoculated littermates.

The piglets were monitored for clinical signs of disease, the fecal shedding of *L.intracellularis*, and the development of serum IgG antibodies against *L.intracellularis*. Six weeks post-primary inoculation, the 8 pigs maintained on sow 3 were inoculated with a similar number of *L.intracellularis*. Infection was monitored as previously described.

The piglets weaned from all three sows before inoculation with *L.intracellularis* developed relatively severe clinical signs of disease between 2 and 4 weeks pi. All of the piglets shed *L.intracellularis* in their feces between 7 and 28 days pi, and developed serum IgG antibodies against *L.intracellularis* between 21 and 35 days pi.

The piglets that remained on sow 1 and 2 after inoculation with *L.intracellularis* developed relatively mild clinical signs of disease, but all shed *L.intracellularis* in their feces and developed serum IgG antibodies against *L.intracellularis* in a similar temporal pattern of infection to the weaned and inoculated pigs. However, the average serum IgG titres against *L.intracellularis* was more than four times lower in unweaned pigs than weaned pigs at 21, 28 and 35 days pi. It appeared that milk from sow 1 and 2 was not able to protect piglets from *L.intracellularis* infection. However, the ingestion of milk from these sows appeared to reduce the severity of disease in their piglets.

The piglets that were inoculated with *L.intracellularis* before weaning from sow 3 did not become infected with *L.intracellularis*. The ingestion of milk from sow 3 appeared to prevent *L.intracellularis* infection in her unweaned, inoculated piglets. The detection of an immune IgG response in sow 3, from day 0 to 28 of the experiment, suggested that sow 3 had recently been infected with *L.intracellularis*.

The weaned piglets from sow 3 had no demonstrable IgG to *L.intracellularis* despite the sow having been recently infected. This suggests that protection against *L.intracellularis* infection is mediated primarily by IgA antibodies in the sow's milk, rather than antibodies derived from colostrum. *L.intracellularis*-specific IgA in the sow's milk may prevent the entry of *L.intracellularis* into intestinal epithelial cells of piglets.

The three previously inoculated pigs and their five uninoculated littermates on sow 3 were all susceptible to *L.intracellularis* infection on re-inoculation, four weeks after ingestion of milk had ceased. The pigs developed a similar temporal pattern of infection as was described previously.

It seems unlikely that sows are a frequent source of *L.intracellularis* infection for their piglets, as the development and secretion of protective IgA antibodies against *L.intracellularis* in the sow's milk would restrict the window of opportunity for the fecal/oral transmission of infection.

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