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Primary infection protects pigs against re-infection with *Lawsonia intracellularis* in experimental challenge studies

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ABSTRACT

In two separate trials pigs were experimentally infected with *Lawsonia intracellularis* at 5–6 weeks of age followed by antibiotic treatment and resolution of the primary infection and then re-inoculated at 12–13 weeks of age. A treatment-control group of pigs received the primary infection and antibiotic treatment only, and served as control for the antibiotic treatment of the primary infection. A challenge-control group of pigs received the second inoculation dose only at 12–13 weeks of age to control infectivity of the challenge-dose and susceptibility of pigs to *L. intracellularis* at this age. Pigs were monitored for shedding of *L. intracellularis* in faeces by PCR, and for the development of antibodies and responses of acute phase proteins in serum. The presence of *L. intracellularis* antigen in the intestinal mucosa was examined in *post mortem* samples by immunohistochemistry.

In both trials primary infected pigs were protected from infection after challenge inoculation as evidenced by absence of faecal shedding of *L. intracellularis*, lack of changes in acute phase protein concentrations after challenge and with low levels of bacterial antigen in the intestinal mucosa of re-inoculated pigs comparable to that of the treatment-control pigs. In contrast, challenge-control pigs shed *L. intracellularis* in faeces, had *L. intracellularis* antigen extensively present within all layers of the intestinal mucosa and developed a significant acute phase protein response in serum after the experimental infection. The acute phase protein response to *L. intracellularis* infection was detected as an increased rise in the serum concentrations of C-reactive protein and haptoglobin from day-6 post infection, and increased serum concentrations of haptoglobin were generally seen 2–3 weeks after inoculation both at 5–6 and 12–13 weeks of age.

In conclusion substantial protection against *L. intracellularis* infection was found in the re-inoculated pigs in contrast to the development of infection in age-matched control pigs. The acute phase protein responses reflected both the observed protection against *L. intracellularis* infection upon secondary challenge and that increased resistance to the infection develops with age.

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1. Introduction

Lawsonia intracellularis is an obligate intracellular bacterium that causes proliferative enteropathy (PE) in pigs. The infection is common worldwide, and in Denmark *L. intracellularis* can be detected in most pig herds. However, infection with *L. intracellularis* does not always cause disease. Generally, weaned pigs and growing pigs develop disease and clinical signs include diarrhoea, uneven weight gain and poor performance. In pigs more than 12 weeks old the infection can cause a more acute hemorrhagic diarrhoea and sudden death (McOrist and Gebhart, 2006). At herd-level PE is mainly controlled by antibiotic treatment and vaccination with an attenuated

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live oral vaccine Enterisol®Ileitis vet., Boehringer Ingelheim (Jacobson et al., 2010). The economic losses caused by PE due to uneven weight gain, mortality, medication and/or vaccination are high (McOrist, 2005).

Ability to induce protection against *L. intracellularis* infection has been investigated by oral administration of the vaccine strain (Kroll et al., 2004), in a field evaluation of the *L. intracellularis* vaccine (McOrist and Smits, 2007) and in a re-infection study by Collins and Love (2007). Some protection was shown by reduced faecal shedding of *L. intracellularis* and reduced scores of specific lesions at post mortem examination in these studies. However, in the study by Collins and Love (2007) age matched pigs were not used to control infectivity of challenge inoculation.

The overall aim of the present study was to investigate protection against *L. intracellularis* infection in age matched pigs using an experimental re-infection model. In two independent experimental infection trials we found a substantial and reproducible protection against challenge infection with *L. intracellularis* compared to development of infection with shedding of bacteria and significant acute phase protein response in serum of age-matched pigs receiving a primary infection. The acute phase protein response is an unspecific indicator of disease and can be measured as an increase in serum concentration of e.g. haptoglobin and C-reactive protein (CRP) in pigs (Petersen et al., 2004). To our knowledge the current data are the first to demonstrate an acute phase protein response to *L. intracellularis* infection and disease.

2. Materials and methods

2.1. Animals and experimental design

A total of 62 pigs (Danish Landrace/Yorkshire/Duroc) of mixed sex were used. The pigs used in Experiment I and II, respectively, came from two different high health and SPF herds both negative for *L. intracellularis* after a medicated eradication program as shown by routine diagnostic testing on faecal samples by PCR and serum samples by ELISA. Additionally the herds were negative for *Salmonella* according to the Danish surveillance program and *Brachyspira hyodysenteriae*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, toxin-producing *Pasteurella multocida*, PRRS virus, lice and manges according to the

SPF program. Cases of other infections in the herds that could interfere with the experiments were not stated.

At 4–5 weeks of age (weight 8–12 kg) pigs were randomly grouped and housed in separate and isolated pens with concrete slatted floor, straw bedding and *ad libitum* access to water. Pigs were fed standard pelleted growing feed, and were allowed to acclimatize for one week before starting the experiment. During this period faeces and blood were collected, and PCR and ELISA diagnostic testing confirmed the absence of *L. intracellularis* infection. Two separate experiments (I and II) with similar experimental designs were performed, however, size of inoculum, number of samplings and end-point of the experiments were different. A schematic overview is shown in Table 1.

Experimental infections with *L. intracellularis* were induced by an oral challenge model, using *L. intracellularis* infected intestinal mucosa isolate from pigs with PE (Boesen et al., 2004). Age-matched non-inoculated pigs served as negative controls.

Pigs in the Re-infection (Re-i) and Treatment Control (TC) groups were inoculated with *L. intracellularis* at day 0, while pigs in the Challenge Control (CC) group were uninfected. At day 21 all pigs in all groups were treated with tiamulin (Tiamutin® Vet.) to clear the *L. intracellularis* infection. Tiamulin was administrated in the feed (20 mg tiamulin/g) for 7 days in both experiments and was in Experiment I preceded by a 3-day intra muscular injection treatment (20 mg/kg pig) to treat clinical infection. Uninfected control pigs also received the antibiotic treatment in order to equal any effects related to this treatment. In the beginning and at the end of the treatment period the pens were cleaned thoroughly with high-pressure water and subsequently disinfected with a broad spectrum disinfectant (2% solution of Virkon® S, Antec International Limited, Suffolk, UK).

Following a three-week resolving phase after antibiotic treatment, a challenge *L. intracellularis* inoculation was given to pigs in the Re-i and CC groups. Thus, TC pigs acted as controls for effective antibiotic treatment against the primary inoculation and CC pigs were age-matched controls for the challenge inoculation.

In Experiment I, all pigs were killed at day 70/74 (approx. three weeks after challenge). In this experiment, one pig died (day 7), one pig was euthanized and

Table 1
Schematic overview of the experimental design.

Day:	0	21	31	49	Kill day 70-84
Group	Primary infection	Antibiotic treatment	Resolving/healing		Challenge infection
Re-infection (Re-i)	/////	||||			/////
Treatment control (TC)	/////	||||			
Challenge control (CC)		||||			/////

Table 2
Group identity and number of pigs in the groups.

Group	Code and identification in figures		Final number of pigs	
			Experiment I	Experiment II
Re-infection	Re-i	■	8	10
Challenge control	CC	□	10	8 (7[a])
Treatment control	TC	▨	4	5

Numbers of pigs in each group at the end of the experiments. In addition nine *L. intracellularis* inoculated pigs and five non-inoculated control pigs were killed during the experiment (Experiment II, day 19/20), data from these pigs are included in the respective groups until death. Furthermore three pigs from Experiment I were excluded from the whole experiment and one pig[a] from Experiment II was excluded from day 49 and forward. For details see Section 2.1. Codes used for identification of pig-groups in the paper are shown in the table. Before day 48, time of challenge inoculation, pigs were grouped together as *L. intracellularis* inoculated pigs (●) and non-inoculated control pigs (○).

necropsied (day 55) because of no weight gain, and one pig revealed chronic fibrinous polyserositis unrelated to *L. intracellularis* infection at necropsy at the end of the experiment. These three pigs were excluded from the whole experiment. In Experiment II, nine primary inoculated pigs and 5 non-infected control pigs were killed (at day 19/20) nearly three weeks after inoculation to measure extent of early onset of *L. intracellularis* infection after the primary infection. The rest of the pigs were killed at day 83/84 (five weeks after challenge). In this experiment one CC-pig died at day 54 due to non-intestinal disorder (acute interstitial pneumonia). Faecal and blood parameters of this pig until death are included in the study (Table 2).

The experiments were conducted in accordance with the guidelines laid down by the Danish Animal Experiments Inspectorate under the Ministry of Justice.

2.2. Preparation of inoculum and inoculation of pigs

Inoculation material was prepared from frozen ($-80\,^\circ$C) severely PE affected porcine small intestines as previously described (Boesen et al., 2004). The concentration of *L. intracellularis* in the inoculum was quantified by indirect immuno-fluorescence as described by Jensen et al. (1997) using an anti-*L. intracellularis* monoclonal antibody (Law1-DK) (Boesen et al., 2005a).

In Experiment I, pigs were fasted from the previous evening and inoculated by oro-gastric tube with 25 ml of inoculum. The pigs received approximately 10^{10} *L. intracellularis* per pig at day 0 and at day 49.

In Experiment II, pigs were fasted from the previous evening and inoculated per oral (allowing the pigs to swallow the inoculum) with 2 ml of inoculum at day 0, approximately 2×10^9 *L. intracellularis* per pig, and 10 ml at day 49, approximately 10^{10} *L. intracellularis* per pig.

The intestinal material used for both experiments was from same batch of small intestines collected from pigs highly infected with *L. intracellularis*. The pigs originated from a Danish fattening herd with low *Salmonella* prevalence according to the Danish *Salmonella* surveillance program. A moderate level of porcine circovirus type 2 (PCV-2) DNA copies were found in the inoculum by PCR (Hjulsager et al., 2009; Jensen et al., 2006), but no differences in anti-PCV-2 titer in serum pre- and post inoculation could be detected in the pigs (data not shown).

2.3. Weight gain and clinical observation

The pigs were weighed at arrival, after the first infection phase, before and three weeks into the second infection phase and at the end of experiment.

Daily observation of performance and faeces fluidity was performed and individual observations that were different from normal observations in healthy pigs were noted.

2.4. Bacteriological examinations

Faecal samples were collected with regular intervals and measured for *L. intracellularis* by real time PCR (Lindecrona et al., 2002). The DNA extract from the faeces samples were diluted 1:100 in Milli-Q water and stored at $4\,^\circ$C until further analysis. Samples of the extracts were run on a RotorGene 3000 (Qiagen, Copenhagen, Denmark) using primers and probes based on the 16S rDNA of *L. intracellularis*.

2.5. Necropsy and immunohistochemical examination

Necropsy was performed around three weeks and five weeks after *L. intracellularis* inoculation. All pigs were subject to necropsy with special emphasis to describe the extent of proliferative enteropathy (PE) and to exclude pigs with other lesions that might have influenced the experimental infection study.

Tissue samples were taken from jejunum (only in Experiment I), ileum, colon and the ileal lymph node for immunohistochemical (IHC) detection of *L. intracellularis* antigenic material using mAb Law1-DK according to Boesen et al. (2004). Blinded samples were scored according to the severity and extension of the lesions: (0) no detection of antigen, (1) focal, (2) moderate, and (3) extensive detection of *L. intracellularis* antigen in epithelium and lamina propria, respectively.

2.6. Measurement of acute phase proteins

Haptoglobin was analysed by a sandwich ELISA using the monoclonal antibody 3.8D7 directed against porcine haptoglobin as described by Sørensen et al. (2006). This ELISA was calibrated against a porcine haptoglobin standard (Saikin Kagaku Co., Ltd, Japan), using a pooled

porcine serum as a secondary standard on all plates. Standard and samples were run in duplicate and the detection limit was 3.3 μg/ml. CRP was determined by a modified version of the sandwich ELISA described by Eckersall et al. (1989), employing a phosphoryl choline coupled dendrimer as the catching-layer and anti human CRP (A0073, DAKO, Glostrup, Denmark) as the detection antibody in a sandwich ELISA (Heegaard et al., 2009). As in-plate standard was used a porcine serum pool previously calibrated using a human serum C-reactive protein calibrator (X0923, DAKO). Standards and samples were run in duplicate and the detection limit was 0.2 μg/ml.

2.7. Measurement of serological response

The specific IgG responses to *L. intracellularis* were measured using deoxycholate extracted antigen in ELISA as described by Boesen et al. (2005b). A cut off at 15 OD% for the present study was established by frequency analysis (95% negative) of the non-inoculated pigs from eight weeks of age and older.

2.8. White blood cell count

Alterations in white blood cell (WBC) count in blood samples were measured in fresh heparin stabilized blood samples by automatic counting using AC900, Swelab (Experiment I) or ABC Vet (Vet ABC[TM], ABX, Montpellier, France) (Experiment II) haematology instruments.

2.9. Statistical analyses

Mann–Whitney test was used to test differences between pig groups and between responses on different days. Two way ANOVA with Bonferroni post test was used to compare acute phase protein response in *L. intracellularis*-inoculated pigs and non-inoculated pigs. Student's *t*-test was used to compare weight gain between different groups of pigs after inoculations.

3. Results

3.1. Clinical signs

In Experiment I, clinically signs, with depressed behaviour, reduced food consumption and diarrhoea (semi-solid to watery stools) were observed in four pigs (#3, #11, #19, #22) out of 12 pigs during week 2–3 after primary inoculation with approx. 10^{10} *L. intracellularis* per pig at approx. 5–6 weeks of age. The other inoculated pigs showed only subclinical signs of disease with soft faeces, but with normal behaviour. In Experiment II, only subclinical signs of disease (soft faeces, but normal behaviour) were observed in three pigs (#27, #37, #52) out of 24 pigs after primary inoculation with approx. 10^9 *L. intracellularis* per pig while all other pigs were without any signs of disease.

After challenge inoculation with 10^{10} bacteria per pig at approx. 12–13 weeks of age, no signs of clinical or subclinical disease were observed in any of the two experiments in neither the re-inoculated nor the challenge control pigs.

3.2. Faecal shedding

Results of faecal shedding are presented in Fig. 1. During 3–4 weeks after primary *L. intracellularis* inoculation at day 0, at least one PCR positive faecal sample was detected in all pigs in Experiment I, and in all but one (#43) of the pigs in Experiment II. Among the nine *L. intracellularis* inoculated-pigs killed after three weeks (day 19/20 p.i.), *L. intracellularis* was not detected in faecal samples in four (#40, #50, #60, #63) of these pigs, however *L. intracellularis* was detected by IHC at necropsy (Fig. 2B). In both experiments the antibiotic treatment stopped the faecal shedding of *L. intracellularis* and the TC group stayed PCR negative for *L. intracellularis* for the rest of the experiment. After challenge inoculation, faecal shedding of
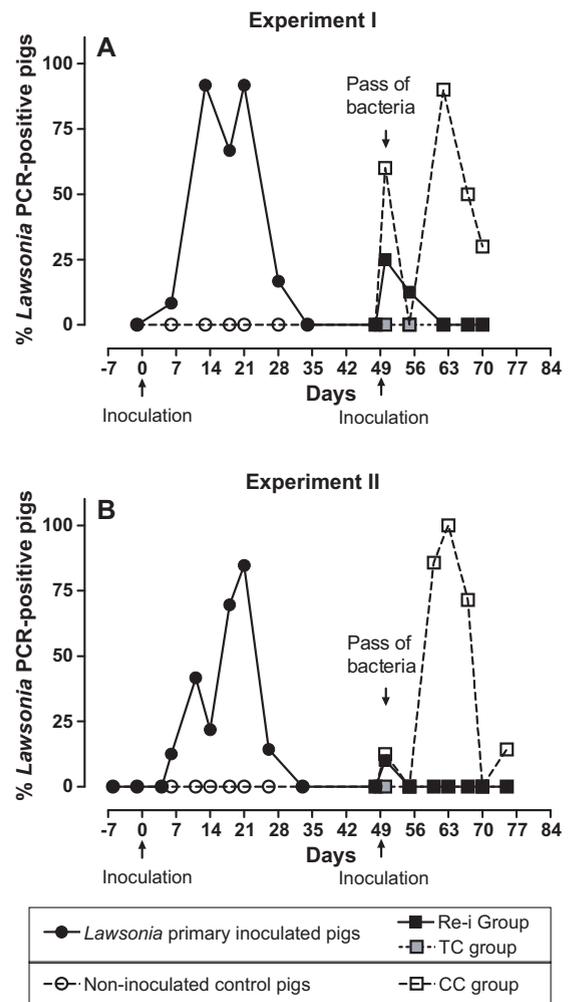
Fig. 1. PCR-detection of *L. intracellularis* in faecal samples. *L. intracellularis* was measured in individual faecal samples and the ratio of pigs with positive *L. intracellularis* detection was calculated. Before day 48, the TC-pigs and the Re-i pigs were grouped together (●) as pigs receiving primary *L. intracellularis* infection, the pigs in the CC-group were in this period non-inoculated control pigs (○). In Experiment II, data from the nine *L. intracellularis* infected and five non-inoculated control pigs, killed at day 19/20, were included from day −7 to day 18.

Experiment I: A
IHC examination 3 weeks after challenge inoculation

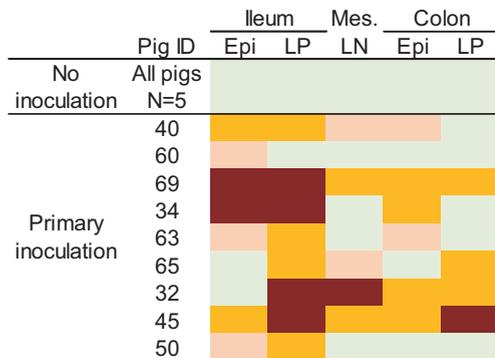|	Pig ID	Jejunum Epi	Jejunum LP	Jejunum LN	Ileum Epi	Ileum LP	Ileum LN	Colon Epi	Colon LP
Re-i	5								
	7					1			
	10		1				2		
	13		1	1					
	18								
	19		1			1	2		
	20		1			2	2		1
	22					1			
TC	3		1			1			
	11						2		
	14		1	1		1			
	16								
CC	1		1		1	1		1	
	2			1		1	1	1	
	6				1	1	2	2	
	9					2			
	12		1	1		2			
	15		1	2	2	3			
	17			1		2			
	21								
	24		1	3	3			2	
	25					2	1		
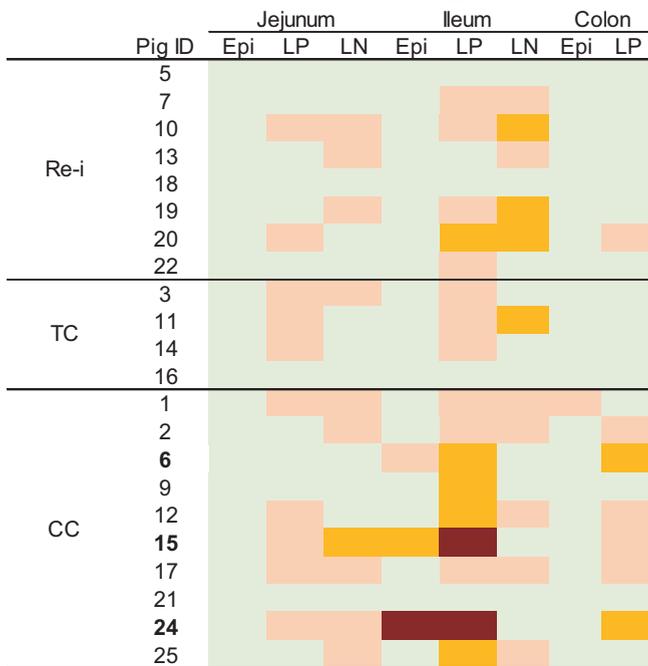
Experiment II: B
IHC examination 3 weeks after primary inoculation

	Pig ID	Ileum Epi	Ileum LP	Mes. LN	Colon Epi	Colon LP
No inoculation	All pigs N=5					
Primary inoculation	40	1	1	1	1	
	60	1				
	69	3	3	2	1	1
	34	1	2	2	2	1
	63		2	1		
	65		2	2	1	
	32		3	3	1	
	45	2	2		3	
	50	1	2			

Experiment II: C
IHC examination 5 weeks after challenge inoculation

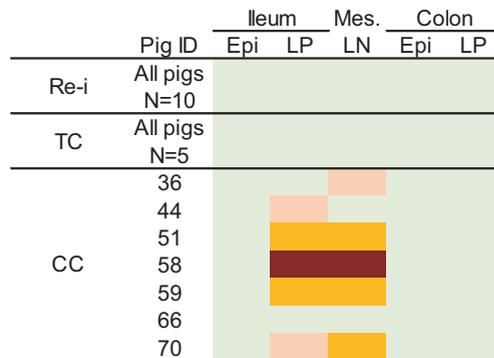|	Pig ID	Ileum Epi	Ileum LP	Mes. LN	Colon Epi	Colon LP
Re-i	All pigs N=10					
TC	All pigs N=5					
CC	36			1		
	44	1	1			
	51	2	2	2		
	58	3	3	3		
	59	2	2	2		
	66					
	70	1	2			

Fig. 2. Scores from immunohistochemical examination for *L. intracellularis* antigen in intestinal tissues. The scoring of the intestines was recorded for epithelium (Epi) and lamina propria (LP) and lymph nodes (LN) separately according to severity and extension of lesions: No staining (= 0), focal (= 1), moderate (= 2), and extensive detection (= 3) of *L. intracellularis* antigen. The identities of individual pigs (Pig ID) in the groups are partly shown in the tables. Pig ID in bold (Experiment I: 6, 15, 24) gross examination revealed PE. The ID of pigs in the group No inoculation (Experiment II, 3 weeks) is: 31, 42, 47, 49, and 67. For Experiment II (5 weeks) the ID of pigs is: Re-I group: 26, 37, 38, 52, 53, 55, 57, 61, 64, and TC group: 27, 29, 43, 62, and 68. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

L. intracellularis was detected in all but one (#21, Experiment I) of the 18 pigs in the challenge control groups in the two experiments. In contrast, faecal shedding of *L. intracellularis* could not be detected in any pigs of the Re-i group. An initial pass of *L. intracellularis* from the inoculum was observed on day 50, the day after challenge inoculation, in both re-infection and challenge control groups.

3.3. Weight gain

In both experiments, some pigs (#3, #11, #16, #19, #20 and #27, #37, #52) showed reduced weight gain during the primary infection compared to the other pigs. For Experiment I this difference was statistically significant ($P < 0.05$, student's t-test) when comparing the primary infected pigs with the uninfected CC pigs from day 0–21. In

Experiment II, where the primary inoculation only resulted in subclinical disease, the statistical difference in mean weight gain from start of experiment until day 21 was not significant ($P = 0.123$). Likewise, comparing mean daily weight gain over the whole study period was not statistically significant between groups in either of the experiments.

3.4. Pathological and immunohistochemical examination

In Experiment I, gross examination revealed PE in 3/10 pigs from the CC group, indicating that the 12 weeks old pigs were susceptible for *L. intracellularis* infection, whereas in the Re-i and TC pigs only minor affections with enteritis or normal appearance without signs of infection was found. Although *L. intracellularis* antigen was detected in the intestines by IHC in nearly all pigs of Experiment I (Fig. 2A) three weeks after challenge inoculation the CC group showed significantly higher total score ($P = 0.0316$, Mann–Whitney) compared to the Re-i group and TC group, which was at a lower but similar level. In Experiment II, *L. intracellularis* antigen was not detected in any pigs in the Re-i group and TC group, five weeks after challenge inoculation, whereas *L. intracellularis* was found in 6/7 pigs in the CC group mainly in lamina propria of ileum and in the ileal lymph node, but not in colon (Fig. 2C). Histopathologically, signs of enteritis related to other infections than *L. intracellularis* were not observed.

3.5. Acute phase protein response

Experiment I: During the first infection period haptoglobin concentrations were significantly higher ($P = 0.0001$) in the group of *L. intracellularis* inoculated pigs compared to the uninfected controls (Fig. 3A). In the uninfected control group a drop in haptoglobin concentration from day −1 to day 6 was observed, presumably indicating accustomization of these animals during this period. In contrast, a substantial rise in serum concentration of haptoglobin was detected at day 13 and persisting to day 21 in the *L. intracellularis* primary infected group. After antibiotic treatment and resolution of the infection both groups converge to an intermediate level (day 41) where after the primary infected group stayed at this level or dropped further at some time points after challenge inoculation (from day 49, Re-i group), showing the same concentrations of haptoglobin as the TC group not receiving challenge inoculation. By contrast, the CC group, showed a rise in serum concentration of haptoglobin after challenge with significantly higher concentrations at day 60 (day 11 p.i.) ($P < 0.01$, Mann–Whitney) compared to the Re-i group. C-reactive protein (CRP) showed the same general changes, although the rise after primary infection occurs already at day 6 and there was a less pronounced drop in the non-infected group during the first period. Also, there was less of a rise in the challenge control group after challenge inoculation at day 49 (Fig. 3B). Neither serum concentrations of haptoglobin nor CRP was significantly increased upon challenge inoculation in the Re-i group (Fig. 3A and B).

Experiment II (Fig. 3C): As observed in Experiment I, an increase in serum haptoglobin concentrations was seen (especially at day 11 and/or 14 in most pigs) in response to primary inoculation with *L. intracellularis*, followed by a decrease. In the same period the haptoglobin concentrations decreased in the group of non-inoculated control pigs. An unexplained slight increase at day 6 was measured in some control pigs. After antibiotic treatment and resolving of the infection, the concentrations of haptoglobin stayed at low level in 4/5 pigs in the TC group until the end of experiment. The serum concentrations of haptoglobin in the Re-i group after challenge inoculation at day 49 were not significantly different from the concentrations in the TC group, however 4/10 pigs (#37, #38, #55, #53) in the Re-i group showed a 2–3 fold higher haptoglobin concentration at day 60 compared to before re-inoculation. In contrast, following inoculation at day 49 the CC group (all eight pigs) experienced increased serum concentrations of haptoglobin, and, compared to the Re-i group, with significantly higher haptoglobin concentrations (Mann–Whitney $P < 0.005$ at day 55–82, except day 60 $P = 0.19$). CRP responses were not analysed in Experiment II.

3.6. L. intracellularis serum antibodies

In Experiment I, all serum samples taken before primary inoculation and of CC group during the 7 weeks before their challenge inoculation, i.e. from the non-inoculated pigs, were sero-negative. In contrast, nearly half of the pigs (45.6%) from Experiment II were ELISA-positive prior to the primary *L. intracellularis* inoculation. None of these pigs were shedding *L. intracellularis* in faeces, and by day 25 (approximately at 8 weeks of age) all the non-inoculated CC pigs were ELISA-negative indicating that these reactions were caused by maternal antibodies.

In response to the primary *L. intracellularis* infection, 7 of 12 pigs (58.3%) in Experiment I and 9 of 13 (69.2%) pigs in Experiment II developed antibody responses above the cut-off. Antibody levels were below cut-off in pigs #5, 10, 16, 19 and 22 of Experiment I and pigs #29, 43, 62, 68 of Experiment II. Of these pigs only #43 was not shedding *L. intracellularis* after inoculation. Although the infection was cleared by antibiotic treatment (from day 21), 12/18 (66.6%) pigs in the Re-i groups were ELISA-positive through day 49 where they were re-inoculated and after which they stayed positive with moderate to high ELISA responses for most pigs and with 15/18 (83.3%) sero-positive pigs at termination. All pigs but one (#17, Experiment I) in the CC groups became ELISA-positive 2–3 weeks after *L. intracellularis* challenge inoculation.

3.7. WBC counts

The effect of *L. intracellularis* infection on the total numbers of WBC in the experiments reflected the severity of the disease. In Experiment I, where the primary *L. intracellularis* infection induced clinical disease in some pigs, WBC counts (10^6/ml, mean ± SEM) were significantly increased ($P < 0.05$, Mann–Whitney) in primary infected pigs at day 13 (20.1 ± 1.6 vs. 13.9 ± 0.9), 18 (21.5 ± 0.9 vs.13.8 ± 0.7) and 21 (22.7 ± 1.6 vs. 16.8 ± 1.0) compared
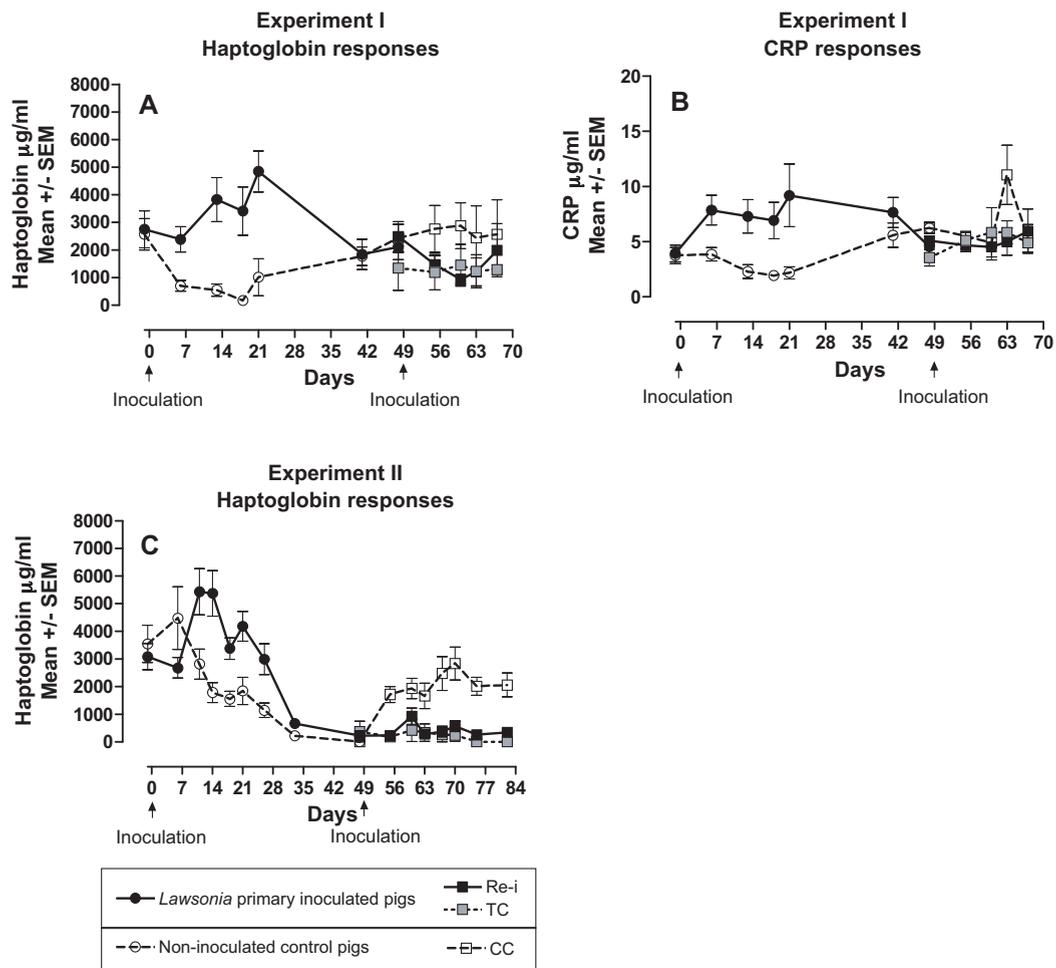
Fig. 3. Acute phase protein responses after primary *L. intracellularis* inoculation and challenge. The levels of haptoglobin were measured in both Experiment I (A) and II (C), whereas CRP responses were only measured in Experiment I (B). Before day 48, the TC-pigs and the Re-i pigs were grouped together as explained in the legends for Fig. 1.

to day −1 (17.1 ± 1.1) and to the non-inoculated controls on the respective days. In Experiment II, where the primary infection only resulted in subclinical infections, no significant differences in levels of WBC were measured between the groups of pigs.

4. Discussion

Challenge inoculation of previously infected pigs did not induce faecal shedding of *L. intracellularis*, whereas pigs challenged without former *L. intracellularis* infection shed *L. intracellularis* from 6 days after inoculation. The present study thus clearly demonstrates that primary infected pigs are protected against re-infection with *L. intracellularis* without shedding of bacteria after the challenge inoculation. Protection against disease and shedding of *L. intracellularis* is important to prevent economic losses due to *L. intracellularis* infection and to increase pig welfare. This study therefore promotes development of effective vaccine strategies to prevent "treatment demanding *L. intracellularis* infection", but also to reduce the spread of *L. intracellularis* within the herd and between herds.

Collins and Love (2007) have previously reported protection with no detection of *L. intracellularis* in faecal samples of pigs challenge inoculated 10 weeks after a primary infection at three different doses (three groups with five pigs per group), whereas the challenge control group (five 4-week old pigs) showed faecal shedding of the bacteria from day 7 p.i. Our data corroborates the conclusions by Collins and Love and, as we used age matched pigs for the challenge inoculation, also excludes the possibility of an age related increase in resistance as sole explanation for their observed protection. Experimental infection with *L. intracellularis* has until now usually been done in 5–8 week old piglets (McOrist et al., 2006) and by induction of PE in the CC pigs the present data show that experimental *L. intracellularis* infection, with development of PE, can be induced in 3 month old pigs. However, although inoculation at 12 weeks of age induced PE, the severity of the infection was lower compared to inoculation at 5 weeks of age with same dose of inoculum. Thus, some age-dependent effect on pathology and level of immune responses prevail,

and age-matched pigs must be used to compare differences between groups.

In Experiment II, we reduced the initial dose of inoculum to develop only subclinical disease in response to primary L. intracellularis infection, which still provided total protection against a challenge infection.

Statistically significant reduction in weight gain after primary L. intracellularis infection was observed only after inoculation of young pigs and only for the pigs in Experiment I, which were also the only pigs to experience significant changes in level of circulating leukocytes. Primary inoculation of older pigs (12–13 weeks of age) did not result in statistically significant changes in weight gain compared to the protected pigs. We did not include a group of pigs that was uninfected throughout the whole study period, and it is therefore not possible to evaluate if the stunted growth after infection in the young pigs was later compensated by increased weight after treatment.

Measurement of serum concentrations of acute phase proteins may be used to detect ongoing disease or injury caused by infective microorganisms (Petersen et al., 2004). In the pig significant increases in the serum concentrations of CRP, haptoglobin, serum amyloid A and pig major acute phase protein occur rapidly after initiation of infection (Petersen et al., 2004). The kinetics of this acute phase protein response is somewhat dependent on the infection in question (Heegaard et al., submitted for publication) and is dependent on the pathogenesis of the infection in question correlating with disturbances in tissue homeostasis. To our knowledge the current data are the first to describe the acute phase protein response to L. intracellularis infection in pigs. Although some variation was seen in the level of responses between pigs, significantly increased serum concentrations of haptoglobin were generally seen 2–3 weeks after inoculation, whereas CRP increased already at day 6 in the young pigs. At day 41 the concentration of haptoglobin was normalised while CRP was still elevated. Interestingly, statistically significant increased serum concentrations of haptoglobin were also observed in Experiment II in which the pigs were only subclinically infected. A significant rise in haptoglobin concentrations was also observed after primary inoculation of the older CC group, albeit smaller than seen during the primary L. intracellularis infection of young pigs while increased haptoglobin and CRP concentrations were not seen in the group of Re-i pigs. The acute phase protein responses thus reflected both the observed protection against L. intracellularis infection upon secondary challenge and that increased resistance to the infection develops with age. Measurement of increased haptoglobin and/or CRP may be used in combination with serological and bacteriological diagnostics to identify "treatment-demanding-disease" caused by L. intracellularis.

Most of the pigs became sero-positive 2–3 weeks after primary inoculation, but in both experiments some pigs were slow responders with a positive response not measured before day 48 p.i., a time where the pigs had been cleared for the infection by antibiotic treatment. Irrespectively of the previous serological status of the Re-i pigs all of them were protected against re-infection as evidenced by absence of L. intracellularis shedding after challenge. In addition, nearly half of the pigs (45.6%) from Experiment II had acquired L. intracellularis specific maternal antibodies, which were not protective against the primary infection in these pigs as evidenced by faecal shedding and increased haptoglobin concentrations after inoculation. These data therefore indicate that passively or actively acquired antibodies against L. intracellularis are not of significant importance in the observed protection against L. intracellularis re-infection.

In conclusion, we observed a distinct and reproducible protection against re-infection with experimental L. intracellularis infection in pigs. This protection was identified by the absence of faecal shedding of L. intracellularis after challenge, low detection of L. intracellularis (low IHC scores) at post mortem examination and a lack of an acute phase protein response after challenge in the previously infected pigs compared to the age matched challenge control pigs. Development of a protective immune response to a mucosal intracellular infection is not surprising and other studies have previously indicated this is true also for L. intracellularis. The current study is, however, the first to include all relevant control groups, whereby a partial age-related resistance to the infection is also documented, and to demonstrate the acute phase protein response in serum as a sensitive indicator of the level of infection. Further studies to characterize the nature of the protection are warranted for a better understanding of protective immune mechanisms and rational development of more effective vaccines against L. intracellularis infection.

Acknowledgements

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