

Studies on the ex vivo survival of *Lawsonia intracellularis*

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Summary

Objective: To examine the ex vivo survival of *Lawsonia intracellularis* in conditions relevant to cleaning procedures on pig farms.

Methods: Two co-cultured strains of *L. intracellularis* were suspended in vitro for 30 minutes at 20°C in various disinfectants according to label recommendations for usage, then washed in saline. Disinfectant-treated and Control bacteria were then added to fresh cells for co-culture for a standard 5-day incubation. Further cell-free suspensions were exposed to air at 5°C, 20°C, or 37°C for up to 8 days, then added to fresh cells for co-culture for a standard 5-day incubation. *Lawsonia intracellularis* within co-cultures were determined to be viable by visualization after indirect immunoperoxidase staining that incorporated specific monoclonal antibody. Groups of weaned pigs were inoculated orally with *L. intracellularis*-infected feces, either on the day the feces were collected, or after storage for 1, 2, or 5 weeks at temperatures between 5°C and 15°C.

Results: Mixing of suspensions of *L. intracellularis* with the quaternary ammonium compound, cetrimide (3.3% wt per vol), for 30 minutes, produced no detectable *Lawsonia* on reculture; mixing with 1% povidone-iodine for 30 minutes produced no or few (< 1% of controls) detectable *Lawsonia* on re-culture. Mixing of suspensions with either 1% potassium peroxy-monosulfate or a 0.33% phenolic mixture for 30 minutes were less effective at the recommended concentrations tested, as small numbers of *L. intracellularis* (1%–18% of controls) were detected on reculture of some strains. Detectable numbers of viable *L. intracellularis* were present after

exposure of cell-free suspensions to air for up to 6 days at 5°C. Colonization of the intestine with *L. intracellularis* was detected in pigs inoculated orally with feces stored for up to 2 weeks at temperatures between 5°C and 15°C.

Implications: *Lawsonia intracellularis* can probably survive in extracellular conditions for 1 to 2 weeks at 5°–15°C. Pure cultures of *L. intracellularis* were fully susceptible to a quaternary ammonium disinfectant (3% cetrimide), less so to 1% povidone-iodine, but not to 1% potassium peroxy-monosulfate or a 0.33% phenolic mixture.

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Porcine proliferative enteropathy (PPE, ileitis) is a common enteric disease affecting pigs raised under various management systems worldwide. The causative agent, *Lawsonia intracellularis*, is a gram-negative, obligate intracellular bacterium in the Desulfovibrio family.^{1,2} Infection of pigs with this bacterium is consistently associated with proliferative mucosal lesions in the ileum and large intestine, which have clinical or subclinical effects on weight gain, feed conversion, and fecal consistency.³ Clinical observations generally include diarrhea and variation in the weights of growing pigs.⁴

Field and challenge-exposure studies indicate that the infection may persist in pigs for at least 10 weeks, with numerous organisms being passed in the feces.^{5,6} Therefore, when any pigs in a group or pen are infected, in-contact pigs are likely to become infected. Preliminary investigations of the on-farm

epidemiology of PPE suggest that infection is most common among 6- to 16-week-old pigs. These weaner-grower pigs, as well as any *L. intracellularis*-infected breeding stock, may play an important role in transmission to younger, susceptible animals via infected feces.^{7–9} Questionnaires and statistical analysis of data from herds with and without pathological evidence of PPE were used to demonstrate a strong association between the occurrence of PPE and the co-mingling of pigs within the previous 30 days.¹⁰ Because there may be a relatively long-term excretion of *L. intracellularis* into pens, information is needed on the possible ex vivo survival time of the organism, and disinfectants that may be effective, to optimize cleaning procedures.

Preliminary pen exposure studies have evaluated infection in sentinel pigs in contact with challenged pigs.^{11,12} Transmission has occurred to pigs housed in pens in the same room, but not to pigs housed in separate rooms. In one study, transmission occurred despite the use of routine cleaning procedures between pens (washing/disinfection of pens, use of footbaths and dedicated boots/clothing).¹² Oral inocula for these and other challenge studies have consisted of cecal contents, or co-cultured pure *L. intracellularis* where available. In the present study, a variety of in vitro and in vivo methods were used to establish the ability of *L. intracellularis* to survive in feces in environmental conditions likely to occur on pig farms.

Materials and methods

Standard co-cultures and monitoring of infection

Three strains of *Lawsonia intracellularis* were used: NCTC 12657 and LRI89/5/83 had been isolated from acute PPE lesions in different 5-month-old British pigs, and the third strain, 51/89, had been isolated from chronic PPE lesions in a 10-week-old pig. The strains were isolated and maintained in co-culture in the rat intestinal epithelial cell line IEC-18 (ATCC CRL

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1589) using methods described previously.¹³ Intestinal material was first inoculated onto cultured cells, and after their initial growth, the bacteria were released from lysed cells, then passaged onto fresh cells. Each isolate used in this study had been passaged between seven and 16 times after initial isolation. During this isolation process, each strain was tested for *Chlamydia* and *Mycoplasma* species and other bacterial contaminants, as described previously.¹³ Quantitative assessment of the numbers of *L. intracellularis* within the cells on each coverslip co-culture was made by counting the number of infected cells after an indirect immunoperoxidase stain that incorporated a monoclonal antibody (IG4) specific to *L. intracellularis*.¹⁴ Challenge-exposure studies have indicated that intracellular bacteria visualized by this staining method are viable and infective for pigs.¹

Evaluation of disinfectant efficacy

Disinfectants selected for testing were:

- potassium peroxydisulfate (oxidizing agent)
- sodium hypochlorite (chlorine-releasing agent)
- phenolic mixture: orthophenyl phenol and orthobenzyl chlorophenol
- povidone-iodine

- hydrogen peroxide and peracetic acid mixture (acidic oxidizing agent)
- cetrimide: a mixture of alkyl trimethyl ammonium bromides (quaternary ammonium agent).

Disinfectant concentrations are shown in Table 1.

Two strains of *L. intracellularis* (NCTC 12657 and 51/89) were freshly harvested from 5-day-old co-cultures and adjusted in cell culture medium to an approximate concentration of 10⁴ organisms per mL. Separate 1-mL suspensions were added to 1-mL volumes of each diluted disinfectant in small plastic tubes, with no organic load present. In preliminary experiments, the recommended concentrations of hydrogen peroxide/peracetic acid and sodium hypochlorite damaged the co-cultures, and a single, lower concentration of each of these disinfectants was tested (Table 1). A solution of 50:50 vol per vol bacteriological medium in water instead of disinfectant was added to control suspensions. Each of the four remaining disinfectants was diluted in sterile, distilled water to the concentration recommended by the manufacturer, and to 0.001 of that concentration (Table 1). Each tube was incubated at 20°C for 30 minutes without agitation, then centrifuged at 12,000 g for 5 minutes, and the

pellet was washed twice in sterile phosphate-buffered saline, pH 7.6. After being resuspended in medium with 7% bovine serum, suspensions were inoculated onto triplicate monolayers which were incubated for the standard 5-day co-culture, after which the coverslips were harvested and the infected cell count established as described above.

Bacterial survival in bacteriological medium in air

Two strains of *L. intracellularis* (NCTC 12657 and LR189) were harvested fresh from 5-day co-cultures, freed from cell material by differential centrifugation, and adjusted in medium to suspensions of approximately 10⁴ organisms per mL. The suspensions were stored separately in plastic vessels at 5°C, 20°C, or 37°C. After 3 hours, 1, 2, 4, 6, or 8 days, 1-mL samples of each suspension were added to fresh, 1-day-old IEC-18 cell monolayers and incubated microaerobically at 37°C in triplicate. After the standard 5-day co-culture, the coverslips were harvested and infected cell counts were determined as described above.

Bacterial survival in feces in air

Feces were collected (Day 0) from an adult pig naturally infected with an Australian strain of *L. intracellularis*. The diagnosis of

Table 1: Effect of various disinfectants on *Lawsonia intracellularis* in a 30-minute suspension assay

Disinfectant tested	Final concentration of disinfectant in cell suspension	Number ± SE of heavily infected cells (>50 organisms per cell) per coverslip (n=3) (relative % compared to control cultures after 5-day co-culture of disinfectant-treated <i>L. intracellularis</i> suspensions)	
		Strain 12657	Strain 51/89
Control medium without disinfectant	0	509 ± 113 (100%)	1163 ± 228 (100%)
Potassium peroxydisulfate	1.0% wt/vol	57 ± 45 ^a (11%)	0 ± 0 ^a (0%)
	0.001% wt/vol	1320 ± 455 ^c (>100%)	1472 ± 101 ^c (>100%)
Phenolic mixture	0.33% vol/vol	Not tested	205 ± 50 ^a (18%)
	0.00033% vol/vol	1029 ± 89 ^a (>100%)	2143 ± 386 ^a (>100%)
Povidone-iodine	1.0% iodine	5 ± 5 ^b (1%)	0 ± 0 ^a (0%)
	0.001% wt/vol	910 ± 224 ^c (>100%)	2328 ± 225 ^a (>100%)
Cetrimide (quaternary ammonium)	3.3% vol/vol	0 ± 0 ^b (0%)	0 ± 0 ^a (0%)
	0.0033% vol/vol	508 ± 212 ^c (100%)	1957 ± 353 ^c (>100%)
Hydrogen peroxide/peracetic acid*	0.0005% vol/vol [†]	931 ± 352 ^c (>100%)	1630 ± 183 ^a (>100%)
Sodium hypochlorite*	0.001% vol/vol [‡]	0 ± 0 ^b (0%)	791 ± 112 ^c (68%)

a concentration differed from control *P* .01

b concentration differed from control .01 > *P* .02

c concentration differed from control *P* .02

* For these disinfectants, concentrations higher than the ones listed, at or near the manufacturer's recommendations, damaged the co-cultures in preliminary studies, and therefore were not tested. The higher of the two concentrations listed for the other disinfectants are based on manufacturers' recommendations.

† Original concentration 23.5% hydrogen peroxide and 4.3% peracetic acid; manufacturer's recommended dilution 1:100 (1% for pigs).

‡ Original concentration 5% sodium hypochlorite; manufacturer's recommended dilution is one part disinfectant to three parts water (10,000 ppm or 0.1% volume:volume final concentration) for farm use.

L. intracellularis infection in the source pig was confirmed by routine pathologic techniques, including histology and indirect immunoperoxidase staining of ileum tissues. Indirect immunoassay of the feces for *L. intracellularis* content, performed as described previously,⁶ indicated between 1×10^6 and 10^7 organisms per g of feces on Day 0. The feces were stored in an open plastic container in a room with a diurnal temperature range of 5°–15°C. Drying of the feces during storage did not allow representative staining and comparisons of fecal smears on Days 7, 14, or 35. Oral inocula were prepared from 10 g of feces resuspended 1:2 vol per vol in sterile phosphate-buffered saline, pH 7.6. Four groups of 3- to 4-week-old pigs were weaned into separate pens in one isolation building, and fed a commercial, wheat-based, pelleted diet, without antibiotics, throughout the trial. There was no direct contact between pigs in different pens, and pigs could not contact the feces of pigs in other pens.

Each of the four groups of pigs was inoculated orally with freshly prepared fecal suspensions so that

- one group (n = 5) received a fecal suspension of fresh feces;
- one group (n = 5) received a fecal suspension of 1-week-old feces;
- one group (n = 3) received a fecal suspension of 2-week-old feces;
- one group (n = 4) received a fecal suspension of 5-week-old feces; and
- a fifth group of unexposed pigs (n = 10) housed in the same isolation building served as controls.

All pigs were monitored for possible signs of clinical infection, including reduced weight gain and diarrhea.

Individual fecal samples were collected from exposed pigs twice weekly and from unexposed controls weekly for PCR amplification of *L. intracellularis* DNA. Total DNA was extracted from 0.2 g of each fecal sample by use of commercial silica-based columns, according to the manufacturer's instructions ("Wizard," Promega; St. Paul, Minnesota). Five μL of each extract was incorporated as DNA template into a PCR reaction designed to detect *L. intracellularis*. We used the PCR reaction mixture, buffers, cycles, and PCR product detection methods of Jones, et al., in a total volume of 50 μL as described elsewhere,¹⁵ except that primers designated LINTf2c and LINT821r were

used. These primers were designed to be complementary to hypervariable regions of the 16S rDNA sequence of *L. intracellularis* (Genbank accession no. L15739). The sequence of primer LINTf2c was 5'-GGGGGAAGAAATGGATGGGC-3' and primer LINT821r was 5'-TTAACTCCCCAGCACCTAGCACC-3'. The accuracy of these primers was verified by confirmatory DNA sequencing of the PCR product from *L. intracellularis* and by their inability to amplify from, or hybridize to, DNA extracted from laboratory isolates of *Campylobacter mucosalis*, *C. hyointestinalis*, *C. coli*, *C. jejuni*, *Escherichia coli* and *Desulfovibrio desulfuricans*, incorporated into our standard reactions. Positive control DNA from cultured *L. intracellularis* (NCTC 12657) was used in each batch of reactions, with a positive PCR product of 402 bp. Blood was collected from all pigs in the study at weekly intervals, and a specific indirect immunofluorescence assay for anti-*Lawsonia intracellularis* IgG was performed on the serum, as described elsewhere.¹⁵

Statistical analysis

Counts for each disinfectant concentration were compared to those of controls by the paired, two-tailed Student T test. Counts for each time point in the air survival study were compared to those of the starting points by the paired, two-tailed Student T test.

Results

Evaluation of disinfectant efficacy

The efficacy of various disinfectants against *L. intracellularis*, as indicated by subsequent co-culture of the organism, is summarized in Table 1. Mixing of cetrimide at the final concentration of 3.3% (wt per vol) in suspensions of the *L. intracellularis* strains resulted in no detectable organisms remaining. Mixing of other disinfectants with the suspensions, at the concentrations tested, resulted in small numbers of at least one strain of *L. intracellularis* being detected in subsequent re-culture, indicating that the disinfectant did not kill all the bacteria in the test suspensions. Mixing of 1% povidone-iodine with *L. intracellularis* did result in complete or marked reduction of detectable bacteria (< 1% of controls) on subsequent re-culture. The acidic oxidizing agent (hydrogen peroxide/peracetic acid) and the chlorine-releasing agent (sodium hypochlorite) considerably damaged the cell monolayers, despite the washing procedure. We

therefore tested reduced concentrations of these agents, which still markedly reduced antibacterial activity: after exposure of strain 12657 to the reduced dose of sodium hypochlorite, no bacteria were detectable on re-culture.

Bacterial survival of bacteriological medium in air

Viable *L. intracellularis* were detectable for up to 6 days in cell-free suspensions kept in culture medium in plastic vessels in air at 5°C, but for only 1 day when kept at higher temperatures (Table 2).

Bacterial survival in feces in air

At the time of oral challenge, no pigs had detectable *L. intracellularis* DNA in their feces, or serum IgG antibodies to *L. intracellularis* (Table 3). Four of five pigs inoculated with fresh feces on Day 0 developed moderate to severe diarrhea beginning 14 days after exposure and persisting for 1 to 4 weeks. One of five pigs challenged with 1-week-old feces developed diarrhea 28 days after exposure, which persisted for 3 weeks. Diarrhea did not occur in other pigs.

PCR products consistent with *Lawsonia intracellularis* DNA were amplified from control DNA material and from the feces of pigs exposed to infected feces after 0, 1, or 2 weeks' storage, but not from pigs exposed to infected feces stored for 5 weeks (Table 3). Similarly, serum IgG antibodies to *L. intracellularis* were detected beginning 21 days after exposure in pigs exposed to infected feces after 0, 1, or 2 weeks' storage, but not in control pigs nor in pigs exposed to infected feces stored for 5 weeks (Table 3). *L. intracellularis* shedding in feces was detected between 5 and 12 days later, and serum IgG antibody development was detected 7 days later, in pigs inoculated with feces stored for 2 weeks compared with pigs given fresh or 1-week-old feces.

Discussion

As *L. intracellularis* cannot be cultured directly from feces, indirect detection methods were employed. The absence of detectable *L. intracellularis* DNA in feces or serum antibodies against *L. intracellularis* at the time of inoculation demonstrated that none of the inoculated pigs had been previously infected with *L. intracellularis*. The similar temporal pattern of shedding of *L. intracellularis* in feces and the development

Table 2: Survival of *L. intracellularis* stored in medium at 5°C, 20°C, or 37°C, for 3 hours to 8 days

Storage time	<i>L. intracellularis</i> strain NCTC 12657 Viable organisms before storage*			<i>L. intracellularis</i> strain LR189 Viable organisms before storage*		
	Control (0 hours)	1038 ± 103			985 ± 80	
	Viable organisms after storage*			Viable organisms after storage*		
	Storage temperature			Storage temperature		
	5° C	20° C	37° C	5° C	20° C	37° C
3 hours	97 ± 19 ^a	110 ± 21	104 ± 18	36 ± 14 ^a	8 ± 4	9 ± 2
1 day	25 ± 14	2 ± 2	0 ± 0	10 ± 8	3 ± 2	0 ± 0
2 days	18 ± 10	0 ± 0	0 ± 0	14 ± 5	0 ± 0	0 ± 0
4 days	16 ± 13	0 ± 0	0 ± 0	18 ± 9	0 ± 0	0 ± 0
6 days	4 ± 2	0 ± 0	0 ± 0	2 ± 2	0 ± 0	0 ± 0
8 days	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

a All values for 3 hours and later are significantly less than the control value ($P < .001$)

* Values expressed as the number ± SE of heavily infected cells (>50 *L. intracellularis* per cell) per coverslip (n=3) after 5-day co-culture of stored or control (0 hours) *L. intracellularis* suspension. Storage medium used was standard cell culture medium described in the text.

Table 3: Challenge studies in pigs inoculated orally with *L. intracellularis*-infected feces stored between 5°C and 15°C for 1, 2, or 5 weeks.

Storage time for fecal inoculum	Occurrence of diarrhea	Positive PCR identified*		Positive IgG titer after challenge†	
		First detected DPI (positive pigs/total pigs)	Last detected DPI (positive pigs/total pigs)	First detected DPI (positive pigs/total pigs)	Last detected DPI (positive pigs/total pigs)
0 hours (5 pigs inoculated)	4/5	7–14 days (5/5)	31–35 days (5/5)	21 days (5/5)	>35 days (5/5)
1 week (5 pigs inoculated)	1/5	7–14 days (5/5)	28–35 days (5/5)	21–28 days (5/5)	>35 days (5/5)
2 weeks (3 pigs inoculated)	0/3	19 days (3/3)	28–35 days (3/3)	28–35 days (3/3)	>42 days (3/3)
5 weeks (4 pigs inoculated)	0/4	undetectable for 42 days (4/4)	undetectable for 42 days (4/4)	undetectable for 42 days (4/4)	undetectable for 42 days (4/4)

* PCR amplification of *L. intracellularis* DNA performed on fecal samples on day of challenge and twice weekly

† Serology performed weekly

of serum IgG antibodies to *L. intracellularis* indicate that no uncontrolled transmission of infection occurred between our trial pigs.

Our observations of in-vitro data and experimental infections of pigs with stored feces suggest that *L. intracellularis* may be viable outside the host for up to 2 weeks under conditions similar to those found on pig farms. The absence of clinical signs and delay of seroconversion and fecal shedding in pigs inoculated with feces stored for 2 weeks suggest that the infectivity of the inoculum might have been reduced. However, previous challenge studies have indicated that only a moderate challenge inoculum (10^6 – 10^8 organisms) is required to initiate pathogenic intestinal infection, and persistent fecal shedding can still occur.^{1,6,16} Our results demonstrating survival and infectivity of *L. intracellularis* in stored feces suggest that fecal contamination of pens, troughs, and equipment could cause a continuing cycle of infection among new pigs, with subsequent fecal shedding of organisms. This emphasizes the need for thorough

cleaning and disinfection of pens and equipment before the introduction of a new batch of pigs. These PCR and serologic studies further corroborate the persistent nature of *L. intracellularis* infections in pigs exposed to infected feces or other inocula.^{5,6} In the absence of adequate biosecurity, spread of infection among groups of pigs in a single building or farm may be rapid and far-reaching.

The ability of some disinfectants to reduce the numbers of *Lawsonia* was partially tested, but further work is needed. Formalin- or glutaraldehyde-based disinfectant compounds were not tested because of their toxicity to humans. Testing methods vary widely, from suspension assays similar to that used in this study, to carrier tests on bacteria placed on a selection of different surfaces. The latter method is not compatible with cell-dependent organisms such as *L. intracellularis*. This preliminary study indicated that quaternary ammonium compounds and iodine may be effective at the recommended concentrations, but phenol disinfectants may be of limited benefit.

Gram-negative organisms have selective membrane porins which are necessary for enteric bacteria to resist toxic effects of fatty acids and bile salts,¹⁷ and which may be responsible for bacterial resistance to certain disinfectants, including phenols. The efficacy of quaternary ammonium compounds may be diminished in the presence of organic material; this factor could not be tested fully in our cell culture-dependent system. The possible causes for variations in infected cell counts at low concentrations of some disinfectants (Table 2) could not be determined. Further work is needed on these aspects of disinfectant activity against *Lawsonia*.

Although effective antibiotics are now known, and on-farm medication programs are available,¹⁸ eradication of *L. intracellularis* infection by medication and livestock control procedures has not been described. Detailed cleaning regimens, including the removal of pigs and feces from pens and the use of disinfectants, should aid disease control in “all-in–all-out” and continuous flow systems. Contact

between the disinfectant and the organism is an important aim of disinfection in a pig farm. Full biosecurity may be achieved only where pens are cleaned and left empty for 2 weeks, and incoming pigs are known to be free of infection. On the basis of our results, we recommend washing pens with hot water to remove all organic material, applying quaternary ammonium- or iodine-based disinfectant for at least 30 minutes before re-washing, then leaving the pens empty for at least 2 weeks. It has been suggested that pens with slatted floors may carry a higher risk of transmission of *Lawsonia*.¹⁹ This may be partly due to relaxed cleaning procedures on these floor systems, but some cleaning procedures, such as high-pressure spray washing, may spread pig feces from slatted floors to adjacent pens during washing. We therefore emphasize that our recommended procedure be applied in a thorough and complete manner.

Our results have shown that *L. intracellularis* has a surprisingly strong capacity to survive outside the host. This may partly explain its common occurrence and apparent ability to persist on pig farms under various management systems. The *ex vivo* survival of *L. intracellularis* in air and feces at 5°–15°C is relevant, since these temperatures regularly occur on farms throughout winter periods. Other studies have shown broadly similar survival patterns (10–20 days) in the microaerobic *Campylobacter fetus* and the anaerobic *Brachyspira hyodysenteriae*, in cultures exposed to air in the laboratory, and in feces. The survival time of these pathogens in infected feces was also enhanced at cool temperatures (5°–10°C) and by dilution in water.^{20,21} The microaerobic *L. intracellularis* retained some viability when stored at 5°–15°C for up to 2 weeks, despite the possibly toxic

effects of atmospheric oxygen and feces. Microaerobic or anaerobic bacteria may be protected within larger portions of feces. In studies with dysenteric feces, *B. hyodysenteriae* could not be isolated from smaller portions of feces air dried at 5°C for 12 hours.²⁰ Eradication programs developed for swine dysentery may be adaptable for proliferative enteropathy.

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