United States isolates of *Lawsonia intracellularis* from porcine proliferative enteropathy resemble European isolates

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Summary

Objective—*To compare isolates of* Lawsonia intracellularis *from United States pigs to European isolates.*

Design and procedure—*Three isolates of* L. intracellularis *were isolated from the intestines of United States pigs with porcine proliferative enteropathy. The organisms were grown and passaged in a rat enterocyte cell line (IEC-18). Growth was not associated with morphological cell change and was monitored by immunostaining of the cells using a monoclonal antibody and by polymerase chain reaction (PCR). The PCR products were evaluated by DNA sequencing and compared to a known sequence from* L. intracellularis.

Results—*Lawsonia intracellularis is a microaerophilic, Gram-negative, curved or S-shaped bacillus and is an obligately intracellular organism.*

Implications—*These isolates resemble European isolates by PCR, monoclonal antibody reactions, and growth requirements.*

Keywords: *swine,* Lawsonia intracellularis*, isolates*

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Porcine proliferative enteropathy (PPE) is a major disease of swine worldwide and continues to be a cause of economic losses in herds, including in Cesarean-derived breeding stock and specific-pathogen-free (SPF) pigs. A consistent disease feature of PPE is the thickening of the mucosa of the small and, sometimes, of the large intestine due to hyperplasia of crypt enterocytes. Curved intracellular bacteria are also consistently present. The prevalence and incidence of PPE are unknown; however, in the United States estimates have been as high as 20% of the swine herd, with losses of $10–$20 million annually.[1]

The intracellular bacteria associated with PPE were previously and incorrectly referred to as "*Campylobacter*-like" organisms. The causative agent has now been identified and is considered to be a novel taxonomic genus and species, previously known by the vernacular name ileal symbiont (IS) intracellularis.[2] Recently, the organism has been given the name *Lawsonia intracellularis* gen., nov., sp. nov.[3] *Lawsonia intracellularis* is an obligate intracellular bacterium and cannot yet be cultivated on conventional cell-free media. Methods for culturing and maintaining *L. intracellularis* have recently been developed in cell cultures.[4] The disease has been reproduced with pure cultures of these bacteria in orally dosed pigs.[5,6] Conventional pigs dosed orally with approximately 3.7×10^6 bacteria passaged six times in cell culture developed severe lesions of proliferative enteropathy and *L. intracellularis* was reisolated from the lesions 21 days after challenge.[5] This study compares *L. intracellularis* successfully cultured from United States pigs to the characteristics previously defined for European isolates.

Materials and methods

Selection of inoculum samples

Sample N24912 was obtained from a herd in Iowa in which 15 of 300 5-month-old finisher pigs were observed to have persistent bloody stools. Penicillin treatment had been attempted. Upon necropsy of the pigs, the mucosa of the ileum was found to be thickened. Histopathology examinations with silver stains demonstrated the presence of curved intracellular bacteria and crypt enterocyte hyperplasia, confirming the diagnosis of PPE.

Sample N72994 was obtained from a 1.5-year-old second-parity SPF sow from a herd in Minnesota. The herd size was between 70–80 sows and antibiotic treatment was unknown. Upon necropsy, the mucosa of the ileum was thickened with some hemorrhage. Giminez staining of the mucosa revealed many curved bacteria.

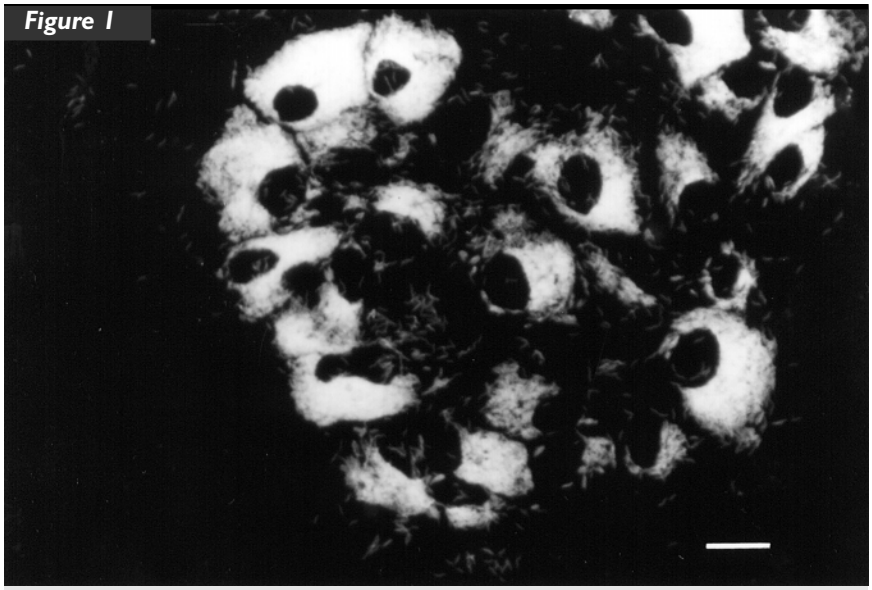
Sample N101494 was obtained from a 12-week-old pig from an

Indiana farm with 600 farrow-to-finish sows. The pig was treated with injectible Tylan® upon the onset of bloody diarrhea, but the pig died soon after treatment.

Preparing the pig-derived bacterial inocula

Intestinal samples were kept at –70°C until processing. Bacteria were isolated by methods previously described.[4] The purified bacterial samples were filtered once through filter paper (Whatman 113V, Whatman Labsales, Hillsboro, Oregon), then sequentially through 5.0-, 1.0-, and 0.65-µm membrane filters. Filtrates were aliquoted and frozen at –70°C in 1-mL aliquots. The mucosa from each case was smeared onto a slide for Giminez stain. Separate smears of filtrates were stained by immunofluorescence assay using a specific monoclonal antibody for *L. intracellularis*[7] as the primary antibody and antimouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate, Organon Teknika Corporation, Durham, North Carolina) as the secondary antibody.

Figure 1



IEC-18 cells infected with N72994, IFA stained using anti-*L. intracellularis* antibodies. Bar = 10µm.

Cell culture

IEC-18 cells (rat intestinal epithelial cells, ATCC CRL 1589) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (JRH Biosciences, Lenexa, Kansas) with L-glutamine and 10% FCS and routinely passaged by trypsin weekly. Cell monolayers were grown at 37°C in air with 5% CO_2.[4]

Infecting the cell culture

The IEC-18 cells were infected as previously described.[4] The cultures were placed in anaerobic jars in which the gas was then replaced with H_2 and CO_2 to give a mixture of 8% O_2, 10% CO_2, and 82% H_2. The cultures were incubated for 3 hours at 37°C, then re-fed with DMEM / 7% FCS with L-glutamine, vancomycin (100 µg per mL), neomycin (50 µg per L), and amphotericin B (2 µg per mL). The cultures were placed back in the anaerobic jars at gas concentrations stated above and incubated for 6 days with media changes every 2 days.[4]

Passage of *L. intracellularis*

Intracellular bacteria were passed by cell lysis using potassium chloride as described previously,[4] then added to fresh IEC-18 monolayers.

Monitoring infection of cell cultures

Infection was monitored by fixing the cells with cold acetone/methanol for 5 minutes. Staining was carried out by immunofluorescence and immunoperoxidase methods. Both methods employed a mouse monoclonal antibody[7] as the primary antibody and either antimouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate, Organon Teknika Corporation, Durham, North Carolina) or peroxidase conjugate (goat antimouse immunoglobulin G, Kirkegaard and Perry Laboratories, Inc.®, Gaithersburg, Maryland). Bacteria were quantified by counting the numbers of specifically stained bacteria within cells on each slide.

Polymerase chain reaction

Sample inocula and passaged bacteria were incorporated as template DNA into PCR using the sample preparation method and primers as described by Jones, et al.[1] The primer set:

5'-TATGGCTGTCAAACACTCCG-3'

and

5'-TGAAGGTATTGGTATTCTCC-3'

was selected for the DNA amplifications. Cycle parameters were 93°C for 5 minutes, 55°C for 45 seconds, and 72°C for 45 seconds for the first cycle. Thirty-three cycles were performed at each of these temperatures for 45 seconds per temperature. The final cycle was 93°C for 45 seconds, 55°C for 45 seconds, and 72°C for 2 minutes. Positive inocula only were used to inoculate IEC-18 cells. Polymerase chain reaction was also performed to monitor passage material to confirm infections. DNA produced by PCR was submitted to the Iowa State University Nucleic Acid Facility for sequencing. Results of the sequencing were compared to sequences obtained by Gary Jones.[8]

Results

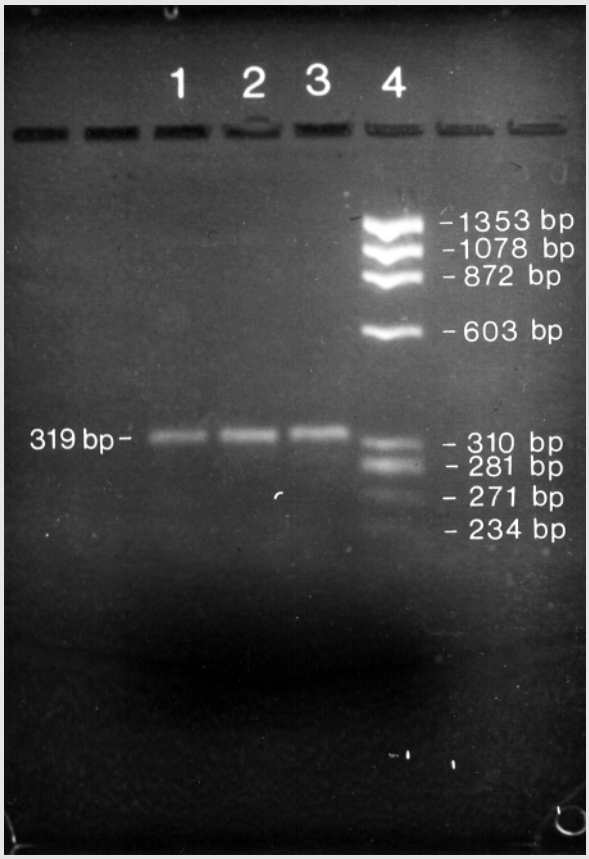
Selecting inoculum samples

Pig numbers N24912 and N72994 had severe proliferative enteritis with bloody intestinal contents and thickened mucosa. N101494 had severe proliferative enteritis and severe hemorrhage resulting in a large blood clot in the intestinal lumen. Giminez staining of the mucosal smears demonstrated large numbers of curved or S-shaped bacteria. Indirect-fluorescent antibody (IFA) assay stains revealed large numbers of brightly fluorescing bacteria in pig-derived bacterial inocula.

Monitoring infection of cell cultures

Inoculated monolayers were monitored by light microscopy throughout the growth cycle and little morphological change of the cells was

Figure 2



Agarose gel of PCR products from isolates grown in IEC-18 cells showing 319 bp fragments. Lane 1, N101494; Lane 2, N72994; Lane 3, N24912; Lane 4, X174 Hae III marker.

observed. Uninfected monolayers grown under reduced oxygen tension (8% O_2) had similar morphology.

Large numbers of curved or S-shaped, specifically stained bacteria, apparently within cells, were observed in immunofluorescence- and immunoperoxidase-stained infected cultures (Figure 1). The monolayers did not have confluent infection. Infected cells were often clumped together, with infected foci of 1–10 cells. Heavily infected cells (cells with 30 or more bacteria) were also seen near cells with fewer than 30 bacteria. Bacterial numbers peaked at or about 6 days.

Infection was dependent on specific growth conditions. A gas mixture of 8% O_2 and 10% CO_2 was necessary to support growth (other gas mixtures were attempted but not successful—data not shown). The bacteria were successfully passaged by the cell lysis procedure.[4]

Polymerase chain reaction

Polymerase chain reaction of chromosomal DNA generated a 319 bp fragment (including primers) from all isolates (Figure 2). A fragment of appropriate size was visually compared to strain 916/91 (NCTC 12657), a known-positive sample generated by McOrist using PCR. Sequence analysis of the PCR products of N24912, N72994, and N101494 confirmed greater than 99% homology to the p78 sequence determined by Gary Jones.[8]

Discussion

This study reports the successful cultivation of isolates of *L. intracellularis* from United States pigs. The current understanding of PPE has been hampered by the fastidious growth requirements of *L. intracellularis*. Porcine proliferative enteropathy has been experimentally reproduced in pigs dosed orally with homogenized adenomatous mucosa originating from United States pigs.[9] This has been effective in causing disease; however, it is difficult to determine the infectious dose or whether other organisms were involved in causing lesions. Reproducing the disease with pure cultures of *L. intracellularis* using European isolates was demonstrated by McOrist, et al.[3] Investigators have been able to reproduce PPE in pigs from a pure culture of the United States strain N72994.[6] This demonstrated that the organism in pure culture could cause disease in pigs after passage in cell culture.

Clinical signs of PPE are often difficult to identify in a field situation. Early clinical signs are often associated with reduced weight gain, most often occurring between 5–12 weeks of age.[10] Previous epidemiological studies have had limited success due to the difficulty of culturing the organism and the limited success of serum testing.

IgA, IgM,[11] and IgG[12] antibodies have been detected in infected animals; however, titers are low or short lived. Lawson, et al., reported that pig sera of known disease status had no IgA response if there was no IgM response.[11] Those that did have an IgA response showed low titers. The IgM response to the bacteria was short lived and persisted for about 8 weeks.[11] As reported by Holyoake, et al., pigs experimentally infected with intestinal homogenates produced low IgG titers in response to infection of *L. intracellularis,* suggesting that IgG plays a minor role in preventing PPE in pigs.[12] To date, serological diagnosis has not been effective due to low antibody titers and short duration. Polymerase chain reaction techniques have been established, allowing a more sensitive detection of *L. intracellularis* in PPE cases.[1,13] Culturing *L. intracellularis* required specialized tissue culture techniques, including a reduced O_2 tension and the use of intestinal epithelial tissue culture cells. The bacteria did not produce cytopathic effects on the monolayers, including cells that were heavily infected. These growth characteristics are similar to the growth characteristics of European isolates reported by Lawson, et al.[4]

Conditions and storage of the intestinal samples were also critical. Ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples were frozen and maintained at –70°C as quickly as possible after necropsy. Samples stored at higher temperatures resulted in reduced infection or no infectivity. If the intestine could not be frozen at –70°C immediately, then it was stored on ice until it was delivered to a facility capable of long-term storage.

The ability to grow isolates of *L. intracellularis* from the United States is a major advance in the understanding of PPE that will allow us to

test for virulence and develop a challenge model, as well as to provide an opportunity to study the pathogenesis of *L. intracellularis* in vitro.

Implications

- *Lawsonia intracellularis* from United States pigs exhibits specialized behavior and fastidious growth requirements similar to European isolates.
- Porcine proliferative enteritis can be reproduced in pigs inoculated with *L. intracellularis* isolated from United States pigs.
- *Lawsonia intracellularis* is a fragile bacteria that must be immediately frozen and maintained at –70°C to ensure infectivity.

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