**In vitro cultivation and immunostaining of *Lawsonia intracellularis* strains**

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**Abstract**

The aim of the study was to implement *in vitro* cultivation of *L. intracellularis* strains using ATCC 55783 and vaccine strains, and McCoy cells (ATCC CRL-1696). The infection was monitored by daily observations under phase contrast microscope. Indirect immunostaining using monoclonal antibody was also performed. Large number of S-shaped, moving bacteria were found in the cell medium in cultures infected with ATCC 55783 and vaccine strain. Immunostaining revealed a high number of multiple cell-associated or intracellular red stained bacteria in the infected cultures. This study describes for the first time *in vitro* cultivation of *L. intracellularis* in Poland, which creates further perspective for more advanced research on this bacterium.

**Key words:** pigs, *Lawsonia intracellularis*, *in vitro* cultivation, McCoy cells, immunostaining.

**Introduction**

*Lawsonia intracellularis* (*L. intracellularis*) is known as an aetiological agent of porcine proliferative enteropathy (PPE), an important disease of swine, affecting growers and finishers (12). PPE is characterised by thickening of the intestinal mucosa at the distal part of the small intestine, due to proliferation of enterocytes related with intracellular bacterial infection. In severe cases, the proliferative lesions may extend to the proximal part of the small intestine or large intestine (12).

Although the disease and bacterium responsible for its development have been known for many years – it was first described in pigs in 1931 (1), there are still certain limitations related to the diagnosis of PPE. The bacterium does not grow on standard media and its *in vitro* cultivation is possible only in cell cultures (6). For this reason, diagnostic laboratories commonly use non-cultivation methods, with necropsy followed by immunohistochemical detection of the bacterium as the gold standard (9, 14).

Currently, only a few laboratories in the world are able to propagate *L. intracellularis* since the method is sophisticated and requires specific conditions. However, for some reasons, like isolation of *L. intracellularis* field strains and their further characterisation, *in vitro* cultivation of this bacterium is the method of choice and cannot be replaced by other technique for the studies of the disease (7).

Up to date, only a dozen isolates have been obtained worldwide (4, 17). Just a few of them were described as being able to develop clinical signs and typical lesions after experimental inoculation (4). Moreover, the availability of these strains still remains very limited (2). For this reason, implementation of *in vitro* cultivation method would increase possibilities to obtain strains from PPE field cases. Before *in vivo* trials, such isolates should be first characterised *in vitro* to estimate possible differences in their virulence.

Therefore, the aim of the study was to develop and to implement *L. intracellularis in vitro* cultivation protocol in the laboratory of the Department of Swine Diseases, as well as to assess strains growth rate and ability to infect cell culture.

**Material and Methods**

**Cell culture.** McCoy cells (ATCC CRL-1696) were grown in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 7% foetal calf
serum (FCS, SIGMA), 1% L-glutamine (Gibco), and 1% amphotericin B (SIGMA), in the atmosphere of 8% O₂, 8.8% CO₂, and 83.2% N₂, at 37°C. The cells were trypsinised at weekly intervals and seeded at a concentration of 0.5 × 10⁵ cells/mL in the cell culture medium.

**Infection of cell culture.** McCoy cell cultures were inoculated with avirulent *L. intracellularis* ATCC 55783 strain and modified-live avirulent *L. intracellularis* strain from a commercially available live vaccine: Enterisol® Ileitis (Boehringer Ingelheim), containing 1×10⁵ – 1×10⁶ TCID₅₀ of the bacterium. The reconstitution of the ATCC 55783 strain was performed according to the ATCC instructions. The freeze dried vaccine was dissolved in 10 mL of sterile water and centrifuged (5000 × g, 10 min). The pellet was suspended in 5 mL of sucrose potassium glutamate (SPG) (5) with 10% foetal calf serum (SIGMA). One millilitre of the suspension was used to infect T-25 flask with McCoy cells of 30% confluency, which corresponded to 24 h culture.

**Monitoring of infection.** The infection was monitored by daily observations of the inoculated cell cultures, as well as the negative control cells under phase contrast microscopy (Optiphot-2, Nikon, Japan, up to 600x). The observations included the assessment of cell culture morphology, as well as search for comma-shaped bacteria actively moving in the cell medium. To estimate the level of infection, indirect immunostaining with monoclonal *L. intracellularis* antibody (Jeno) was performed before each passage (14). Briefly, one loop of scraped cells was spread on a microscope slide, dried, and fixed in methanol. The cells were incubated for 30 min with monoclonal antibody diluted in PBS (1:10), washed with PBS, and incubated for 45 min with the secondary antibody conjugated with HRP – EnVision System Labelled Polymer – HRP Anti-Mouse (Dako). Afterwards, the cells were stained with the enzyme substrate - aminoethylcarbazole (AEC, Dako) for 20 min, washed twice with PBS, mounted, and checked under the microscope (Optiphot-2, Nikon, Japan, up to 600x) for the presence of red-brownish stained bacteria (positive result).

**Passage of *L. intracellularis*.** For the passage of the bacterium, 100% confluent McCoy cells were scraped and lysed by forcing the cell suspension through a 3.5 inch 22 Gauge spinal syringe (Nunc). The suspension was centrifuged at 1500 × g for 10 min to remove cell debris. Then, the centrifugation was repeated at 5000 × g, for 20 min to obtain the bacteria. The pellet was suspended in 3 mL of medium and examined under microscope (Optiphot-2, Nikon, Japan, up to 600x) for the presence of live, moving bacteria and possible McCoy cell leftovers. Afterwards, the suspension was transferred into new flasks (either 25 or 75 cm²) with cell culture and incubated up to 7 d, depending on the growth rate of the bacteria. During each passage the number of microorganisms was counted in the highest countable dilution under light microscopy, in duplicate, as described earlier (4). Six passages were done for each isolate.

**Freezing *L. intracellularis* strains.** The bacteria were harvested as described above. The pellet was suspended in SPG with 10% foetal calf serum and mixed. A drop of the suspension was examined under microscope (magnification up to 600x) to assess morphology and viability of the bacteria. The suspension was then aliquoted into small, screw-cap tubes (Corning) and stored at -80°C until the next usage.

**Results**

Daily observations revealed large number of S-shaped, moving bacteria in the cell medium. The infected cells had a tendency to clump together, creating infected foci of several cells. However, the cells’ morphology was not affected by the infection compared to the negative control cells.

The cell suspension preparations, observed under phase contrast microscopy, showed McCoy cells floating freely in the medium, with the presence of numerous, tiny, rod-shaped, S-shaped, or curved bacteria of *L. intracellularis* morphology. In the subsequent cultures, no visible inhibition of cell growth was observed in flasks containing cells infected either with the ATCC 55783 strain or with the vaccine strain. The average number of bacteria in the flasks with cells infected with ATCC 55783 strain was 1.5 × 10⁸ bacteria/mL, while in flasks containing cells infected with vaccine strain, the average concentration was 3.4 × 10⁷ bacteria/mL. However, these differences may be insignificant, since the initial number of *L. intracellularis* ATCC 55783 remained unknown.

The immunostaining of the infected cell cultures revealed intracellular multiple cell-associated, red stained bacteria, (Figs 1A, 1B). It was especially intense in the case of cells infected with the ATCC 55783 strain, which formed large foci (Fig. 1A). On the contrary, the cells inoculated with the vaccine strain presented less intense staining, suggesting a lower number of bacteria (Fig. 1C). There was a clear difference in the number of intracellular and cells associated bacteria in McCoy cells, infected with ATCC 55783 strain and the vaccine strains. Visibly less microorganisms were seen in the case of the vaccine strain. Non-infected, negative control cells remained unstained and detached from each other.
Discussion

Among various methods for *L. intracellularis* identification, *in vitro* cultivation is still one of the major challenges for diagnostic laboratories due to the specific environmental requirements for the pathogen.

As far as we know, this is the first description of *in vitro* cultivation of *L. intracellularis* in Poland. The technique, described initially by Lawson *et al.* (13), with former modifications by other authors (4, 18), provides the possibility to isolate *L. intracellularis* strains from field cases of proliferative enteropathy and enables to propagate such strains for experimental purposes (i.e. inoculation of pigs). The latter is crucial for the studies on pathogenesis of *L. intracellularis* infection, which to a large extent remains unexplored (8). Besides, the method has a variety of applications. Among them, isolation of *L. intracellularis* strains is indispensable to monitor antibiotic susceptibility, which remains one of the major concerns of the successful therapy of animals (20). *In vitro* cultivation technique also provides antigen for development of quantitative laboratory diagnostic methods, like real-time PCR. Moreover, this method is prerequisite for discrimination of antigenic proteins of *L. intracellularis* isolates (17).

For a long time, *in vitro* cultivation of *L. intracellularis* has been performed in IEC-18, a rat small intestine cell line (13). However, previous studies have shown that many various intracellular bacteria could be detected in the same samples using these cells, therefore the passage of *L. intracellularis* was prone to contaminations and therefore difficult to accomplish (18). For this reason, different cells have been implemented in this technique. Murine fibroblast-like McCoy cells used in this study can be applied for propagation of many microorganisms, which development is dependent on eucaryotic host cell (3). This modified technique improves and simplifies the passage of *L. intracellularis*.

In the study, no cytopathic effect was observed after infection. However, the development of cytopathic effect in *L. intracellularis* infected cells remains controversial. According to Lawson *et al.* (13), heavily infected cells are rounded and detached from the monolayers. Conversely, other authors did not observe any morphological changes in the infected monolayers, even using a high number of bacteria (2, 10, 11, 19).

This study provides information on *in vitro* growth rate and ability to infect cells with *L. intracellularis* ATCC 55783 strain and vaccine strain. For both of them, a fast increase in the number of microorganisms was observed at each passage. Immunostaining also confirmed that both strains were able to infect McCoy cells. However, more intense red-brownish staining, corresponding to higher number of cell-attached or intracellular bacteria, was found in the case of cells infected with the ATCC 55783 strain, compared to those inoculated with the vaccine strain. Similar observations were described by Guedes *et al.* (5), who compared the vaccine strain with field isolate. Moreover, cells infected with the ATCC 55783 strain were accumulated in bigger clumps, while those inoculated with the vaccine strain were congregated in groups of only a few cells. This observation could be explained by the fact that in cell culture heavily infected cells are present in such foci, indicating transfer of infection between cells in its initial phase (17).

As mentioned before, the number of isolated *L. intracellularis* strains is very limited. Currently, *L. intracellularis* ATCC 55783 strain used in this study
is the only commercially available isolate. However, this strain is a patent deposit (Patent No. US 5885823), therefore ATCC has no specific growth information, and no product sheet is available. For this isolate, no laboratory work was performed other than basic viability testing, thus no strain specification is accessible. The only information included in the patent description (Patent No. US 5885823 A), states that the strain is avirulent.

Enterisol Ileitis vaccine (Boehringer Ingelheim Animal Health GmbH) comprises the B3903 strain (ATCC PTA 4926) of *L. intracellularis* (15), which is covered by the patent EP 1651260B1 until 2024 (personal communication - Dr. Bernd Grosse Liesner, Boehringer Ingelheim Animal Health GmbH, 2013). According to the previous studies, *in vivo* inoculation using Enterisol Ileitis as a source of vaccine strain results in slight clinical symptoms of the disease, limited only to the sporadic cases of transient diarrhoea, as well as later and shorter faecal shedding (5). Since both strains used in this study are avirulent, similar outcome could be expected after infection with the ATCC 55783 strain. Both strains are also able to infect cells, and to trigger the development of an immune response (Patent No. US 5885823 A, 6), without causing unnecessary suffering of animals. These data indicates high usefulness of both strains propagated in our laboratory for experimental trials, and confirm the need for new isolates for research purposes.

In conclusion, the described method and the obtained pure cultures of both strains may be used in the further projects, concerning an insight into the pathogenesis and immunology of *L. intracellularis* infections, based on experimental inoculations of conventional pigs.

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