TITLE: An alternative method for cultivation of *Lawsonia intracellularis*

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ABSTRACT
An alternative method for cultivation of *Lawsonia intracellularis*, an obligate intracellular bacterium and causative agent of proliferative enteropathy, was described using Original Space Bag® inflated with a mixture of gas containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen. The flexibility of this protocol allows for the testing of various environmental conditions for static cultivation of this bacterium and the development of diagnostic techniques.

TEXT
*Lawsonia intracellularis* is a fastidious and obligate intracellular bacterium and the causative agent of proliferative enteropathy or ileitis. The disease has been reported in a variety of animal species including nonhuman primates, but it has been best described in hamsters, pigs and horses (2). Dividing eukaryotic cells in culture and strict environmental conditions are required for isolation and cultivation of *L. intracellularis in vitro* (6). The conventional method for isolation and cultivation of this bacterium in monolayer is well established using various methods of supplying hydrogen for infection followed by incubation in a Tri-gas incubators with 83.2% nitrogen, 8.8% carbon dioxide and 8% oxygen at 37°C (3, 8). The cost of these requirements has limited the maintenance of this microorganism *in vitro* to only a few research institutes. Furthermore, since this disease was first reported (1), there have been only a dozen or so cultured *L. intracellularis* isolates worldwide so far. This study describes an alternative method for cultivation of *L. intracellularis* in cell monolayers providing necessary atmospheric conditions for growth without Tri-gas incubators. This alternative protocol presents new opportunities for testing different environmental conditions for isolation and cultivation of this organism. Additionally, it also allows the development of diagnostic approaches including
immunoperoxidase monolayer assay, indirect immunofluorescence and minimum inhibitory concentration.

The *L. intracellularis* isolate PHE/MN1-00 (ATCC PTA-3457) previously isolated from a pig with the hemorrhagic form of proliferative enteropathy was used for evaluating its growth and *in vitro* infection under two different environmental conditions (conventional and alternative). This isolate was grown in murine fibroblast-like McCoy cells (ATCC CRL 1696), maintained in a cell culture system and stored at -72°C until use, as described previously (3). Frozen bacteria were thawed and grown in cell culture for three continuous passages in order to allow the bacteria to recover from the frozen stage. In these three passages, the bacteria was grown using both the conventional and alternative method (Figure 1), as described below. The infection was monitored during every passage using immunoperoxidase staining with polyclonal antibody specific for *L. intracellularis* (3). After three passages, 16-well, glass-bottom tissue culture plates containing one-day-old McCoy (30% confluence) cells were infected (Day 0) with bacterial suspensions containing approximately $10^4$ *L. intracellularis* organisms/well. Murine fibroblast-like McCoy cells were grown in Dulbecco’s Modified Eagles Medium (DMEM; Gibco Invitrogen Corporation) with 1% L-glutamine (Gibco Invitrogen Corporation), 7% fetal bovine serum (FBS; Sigma Chemical) and 0.5% amphotericin B (Cellgro; Mediatech), without antibiotics or medium replacements throughout the study (3). The infected cells were incubated using two different methods. For the conventional method, the cells were placed in the Tri-gas incubator with 83.2% nitrogen gas, 8.8% carbon dioxide and 8% oxygen gas and a temperature of 37°C (6). Tissue culture plates were removed and flushed with hydrogen gas daily. For the alternative method, the plates were placed in an Original Space Bag® (Storage Packs, San Diego, CA, USA,
measuring 54 cm x 85 cm which was hermetically closed. The air inside the bag was then removed by vacuum pump to a pressure of 100 mm Hg. Afterward, the bag was inflated through a cuff containing a 0.22 µm filter connected to a gas cylinder containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen gas. Finally, the bag was incubated at 37°C for eight days (Figure 1). The atmosphere inside the bag was replaced as described above every 24 hours. Carbon dioxide and oxygen gas percentages were monitored in both protocols using CO₂ and O₂ indicators (FYRITE® Gas Analyzer) at the initiation of incubation and every 24 hours during the eight days. The CO₂ and O₂ levels were demonstrated to be stable in the bag when they were measured daily after inflating and before replacing the gas mixture. The CyQuant® cell proliferation assay, a fluorescence-based approach for determining numbers of cultured cells (5), was used to monitor the cell growth of infected and non-infected cells for both incubation methods. In addition, the population doubling time (days) of the McCoy cells was calculated using the algorithm provided by http://www.doubling-time.com (11), based on the intensity of the fluorescence demonstrated in the CyQuant® assay. Infection and growth monitoring of *L. intracellularis* were performed by direct counting of heavily infected cells (HIC), identified by immunocytochemistry staining with polyclonal antibody specific for *L. intracellularis* (3), and by quantitative PCR (qPCR), as previously described (10). The level of infection was also monitored by calculating the estimated population doublings of the HIC, which were previously counted. The number of IHC and the number of *L. intracellularis* organisms were quantified using four replicates (wells) in 16-well tissue culture plates. The average from four replicates was used in the statistical analysis. Wilcoxon signed-rank test was performed using SAS software (9.1) to assess differences between both incubation methods. A value of *p*<0.05 was considered significant.
During the eight days of incubation, the carbon dioxide and oxygen gas were constant (8.8% CO₂; 8.0% O₂) in the Tri-gas incubator (conventional method). In the alternative method, CO₂ and O₂ levels ranged between 7.0-8.0% and 5.5-6.5%, respectively (Table 1). The CyQuant® cell proliferation assay was used to measure cellular DNA via fluorescent dye binding in non-infected and infected cells every 24 hours for eight days of incubation. Non-infected cells had similar growth rates and no significant difference on average of estimated population doubling (calculated by day) between the conventional protocol at 5% CO₂/37°C (1.83±0.18), the Tri-gas incubator (1.79±0.06) and the plastic bag (1.76±0.08). These results showed that non-infected McCoy cells are able to be grown in these bags and able to support the cultivation of L. intracellularis. The bag described previously in this study was able to support up to 12 T₂₅ (Figure 1) or six T₁₇₅ tissue culture flasks (Corning®). However, we have observed that larger bags from the same manufacturer support higher number of flasks.

Enterocyte proliferation is the primary lesion associated with L. intracellularis infection in vivo (2). Although previous experiments have not reported cellular proliferation in vitro (7, 9) to date, there is no information regarding the cell growth during in vitro infection. Using the CyQuant® cell proliferation assay, the growth curves of infected and non-infected cells were compared for the conventional and alternative methods (Figure 2). The results did not show statistical differences (p<0.05) between infected and non-infected in either incubation method. Oh et al (2010) described up-regulation of cell cycle genes in infected McCoy cells; however, cell growth was not measured in this experiment. Furthermore, epithelial growth factors and their interactions with the lamina propria during in vivo infections can play a critical role in the pathogenesis of the disease, which is still poorly understood.
The number of heavily infected cells increased progressively and reached the peak on day seven post-infection in both the conventional and alternative incubation methods. There was no significant difference \( (p<0.05) \) in the number HIC throughout the days of incubation (Figure 3).

No significant difference was found in the estimated population doubling of HIC using the conventional \((2.8 \pm 0.2)\) and the alternative \((3.1 \pm 0.3)\) protocols. Similar to the immunocytochemistry results, the greatest number of \( L. \) intracellularis organisms per well was observed on day seven post-infection by quantitative PCR (Figure 3). In addition, the quantitative PCR showed no significant difference between the conventional and alternative methods of incubation. There was no positive reaction for the non-infected cell cultures (negative control) in the qPCR. Previous studies have described \( L. \) intracellularis cultivation in five to seven days using a Tri-gas incubator, including experiments to validate diagnostic approaches (4) and to investigate the pathogenesis of proliferative enteropathy (9). However, these studies failed to quantify the numbers of \( L. \) intracellularis organisms or the number of HIC in the infected cultures.

Similar to the conventional method, incubation in the bag provided environmental conditions that enable \( L. \) intracellularis to infect and multiply in the cells. Based on these results, we believe this approach can be used for the static cultivation and, potentially, isolation of this bacterium without requiring a Tri-gas incubator. In addition, our experience has shown no difference in the cell growth or level of the infection when the gas inside the bag is replaced at least two times (on second and fifth days post-infection) throughout the period of incubation. This alternative method has been also successfully reproduced (J. S. V. Oliveira and R. M. C.)
Guedes, unpublished data) in an independent trial. This fact has confirmed the usefulness and feasibility of the present method. The flexibility of this methodology allows for the testing of various environmental conditions for *L. intracellularis* cultivation and production of antigens for the development of diagnostic techniques. Additionally, this affordable technology gives to research institutes an opportunity to explore this bacterial proliferative disease, which has intriguing and unique properties among bacterial pathogens.

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REFERENCES


Table 1. Carbon dioxide and oxygen gas levels during eight days of incubation in a conventional Tri-gas incubator and the Original Space Bag®.

<table>
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Figure 2: Quantification of infected and non-infected cells in the TRI-gas and Bag incubation systems using CyQuant® cell proliferation assay. Fluorescence intensities are based on cellular DNA content during days of incubation. Infected cells were measured from day zero (arrow).
Figure 3. Infectious and growth monitoring of L. intracellularis in cell culture plates during eight days of incubation. Direct counting of the number of boundary infected cells by immunocytochemistry (left). Morphology of infected cells in the alternative flag isolation system over time (bottom left). Copies of the separate genomic base pairs of L. intracellularis measured by quantitative PCR (right).