Cultivation and Characterization of *Lawsonia intracellularis* Isolated from Rabbit and Pig

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**ABSTRACT.** *Lawsonia intracellularis* is an obligate intracellular pathogenic bacterium that causes proliferative enteropathy in domestic and experimental animals. In this study, we improved the *in vitro* cultivation method of *L. intracellularis* to increase the passage efficiency and showed that *L. intracellularis* isolated from a rabbit and a pig have different antigenic properties. Bacteria should be recovered from infected cells before cell death due to infection to obtain higher bacterial passage efficiency, and measurement of LDH activity in the cell culture medium was useful for determining the timing of bacterial passage. *L. intracellularis* isolated from the rabbit and pig showed different band patterns in immunoblotting. Our results should be helpful in the development of serological diagnosis and epidemiological investigation methods.

**KEY WORDS:** *Lawsonia intracellularis*, rabbit, swine.

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Proliferative enteropathy (PE) is an intestinal infectious disease characterized by thickening of the aboral small and proximal large intestinal mucosa due to enterocyte proliferation associated with the presence of an intracellular bacterium [7]. The obligate intracellular bacterium, causative agent of PE, has been characterized as a novel genus and species and named *Lawsonia intracellularis* [5, 6]. *L. intracellularis* has been associated with colonization of enterocytes in rabbits, hamsters, rats, guinea pigs, swine, sheep, horses, white-tailed deer, dogs, arctic fox, ferrets, and ostriches [1, 3, 11]. *L. intracellularis* isolated from pigs suffering from PE have been cultivated *in vitro* using IEC-18 cells, a rat small intestinal cell line [5]. Previously, we reported on the *in vitro* cultivation of *L. intracellularis* from rabbits with PE by the same method, and while many intracellular bacteria could be detected in the IEC-18 cells, it was difficult to accomplish the passage of *L. intracellularis* by this method [8, 9]. In the present study, we describe a modified version of the *in vitro* cultivation method in which McCoy cells were used. This improved bacterial passage enabled us to observe differences between the antigenic proteins of *L. intracellularis* isolated from a pig and a rabbit.

Fecal samples were collected from a six-week-old female New Zealand White rabbit and five-month-old female pig with diarrhea, and tested for *L. intracellularis* DNA by PCR [4] to check for infection by it. As the fecal samples were positive, the rabbit and pig were suspected of being infected by *L. intracellularis*. Thickening of the gut wall was also observed. To confirm infection by *L. intracellularis* and improve the method we used previously, we cultivated *L. intracellularis* by the McCoy cell culture method [10]. Briefly, frozen (−80°C) intestine was homogenized and used as the source material. Mouse fibroblasts (McCoy cells; ATCC CRL 1696) were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma) with 10% fetal calf serum (FCS) on glass coverslips on a 6-well plate. Monolayers were exposed to the source material and then centrifuged at 700 × g before transfer to an incubator. The incubator was kept in an atmosphere of 8.0% O2 and 8.8% CO2 at 37°C. The culture was incubated for 6 hr, and the medium was replaced with DMEM containing gentamicin (50 µg/ml) for killing extracellular bacteria. Then, the culture was incubated for 18 hr, and the medium was replaced with DMEM. At 3 to 6 days after infection, on examining the infected monolayer by phase microscopy, there was little morphological change, but several detached cells were observed. *L. intracellularis* was detected by immunohistochemistry with anti-*L. intracellularis* rabbit antiserum [8, 9]. Infected cells were fixed with 4% paraformaldehyde and then permeabilized in 0.1% Triton X-100. Samples were stained with the antiserum for 1 hr at 37°C and after washing three times for 5 min in phosphate-buffered saline (PBS), they were stained simultaneously with FITC labeled anti-rabbit IgG. Samples were placed in mounting medium and visualized by fluorescence microscopy.

In the case of *L. intracellularis* isolated from the rabbit, many intracellular bacteria reacted with anti-*L. intracellularis* antiserum at 4 days after infection, but not at 3, 5 and 6 days after infection (Fig. 1). The same result was obtained for *L. intracellularis* isolated from the pig (data not shown). Next, we attempted passage of *L. intracellularis* using the bacteria recovered from the McCoy cells at 3 to 6 days after infection. Passage was successful with the bacteria recovered at 4 days after infection (Fig. 1). The same result was obtained for *L. intracellularis* isolated from the pig (data not shown). The peak of intracellular growth of *L. intracellularis* under these conditions should be 4 days after infection, and then the McCoy cells would die from the bacterial infec-
To improve the passage efficiency, the activity of LDH released into the culture medium by *L. intracellularis*-infected McCoy cells was measured in order to determine cell viability using an LDH-Cytotoxicity Assay Kit (MBL, Nagoya, Japan) from 1 to 7 days after infection. The absorbance was measured at 492 nm by an ELISA reader (model 450, Bio-Rad, CA, U.S.A.). At 5 and 6 days after infection, LDH activity in the culture medium was much higher than that of the uninfected control (Fig. 2). This result indicated that bacteria should be recovered from the infected cells before cell death, which was 4 days after infection under these conditions, to obtain higher bacterial passage efficiency. Thus, measurement of LDH activity in the cell culture medium is useful for determining the timing of bacterial passage.

We next investigated the differences in antigenic properties between *L. intracellularis* isolated from the rabbit and pig by cultivating the bacteria in McCoy cells. Bacteria recovered from the infected McCoy cells were suspended in SDS-PAGE sample buffer. Samples were analyzed by

Fig. 1. Fluorescence micrographs of intracellular replicated *L. intracellularis*. McCoy cells were infected with *L. intracellularis* from rabbit intestine sample (primary culture) and passage (first passage), and incubated for the indicated number of days. On examination, they were stained with anti-*L. intracellularis* antiserum.
means of SDS-PAGE and immunoblotting with anti-\textit{L. intracellularis} mouse antiserum. The result showed that several proteins from both \textit{L. intracellularis} isolated from the rabbit and pig reacted with the anti-\textit{L. intracellularis} antiserum, but not with normal control serum (Fig. 3). We noted that the several antigens manifested in the \textit{L. intracellularis} isolated from the pig were the same as those seen in a previous study [2], but the \textit{L. intracellularis} isolated from the rabbit did not produce the same band pattern as that from the pig (Fig. 3). These results suggested that \textit{L. intracellularis} isolated from the rabbit and pig might have different antigenic properties and showed more helpful in the serological typing, diagnosis, and epidemiological study of \textit{L. intracellularis} isolated from pigs and other animals.

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REFERENCES