NOTE *Bacteriology*

Enzyme-Linked Immunosorbent Assay to Detect *Lawsonia intracellularis* in Rabbits with Proliferative Enteropathy

Masahisa WATARAI[1)], Yosuke YAMATO[1)], Noriyuki HORIUCHI[2)], Suk KIM[1)], Yoshitaka OMATA[3)], Toshikazu SHIRAHATA[1)] and Hidefumi FURUOKA[2)]

[1)]*Departments of Applied Veterinary Science,* [2)]*Pathological Science and* [3)]*Basic Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*

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ABSTRACT. *Lawsonia intracellularis* is an obligate intracellular pathogenic bacterium that causes proliferative enteropathy in domestic and experimental animals. Antiserum against synthetic peptides of the Lawsonia surface antigen (LsaA) well recognized *L. intracellularis* in infected ileum by immunohistochemistry. The synthetic peptides in LsaA showed strong reaction with serum from rabbits infected with *L. intracellularis* by enzyme-linked immunosorbent assay. These results suggest that ELISA used synthetic peptides in LsaA and anti-LsaA serum might be useful to diagnose for proliferative enteropathy.
KEY WORDS: ELISA, *Lawsonia intracellularis*, rabbit.

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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 [11]. This obligate intracellular bacterium, the causative agent of PE, was characterized as a novel genus and species and was named *Lawsonia intracellularis* [10]. *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 ostrich [1, 5, 12]. Because of the development of *L. intracellularis* specific primers [6], detection of fecal shedding by PCR has been possible. Serum IgG specific against *L. intracellularis* has been detected in pigs by an immunofluorescence antibody test [4, 7] or immunoperoxidase monolayer assay [2, 3]. However, serological detection of *L. intracellularis* is not established in Japan. In this study, we report a case of a rabbit infected with *L. intracellularis* in Japan and detection of the bacteria by antiserum for *Lawsonia* surface antigen.

A six-week-old female New Zealand White rabbit showed diarrhea, and fecal samples were collected and tested for *L. intracellularis* DNA by PCR [6] to check the infection of *L. intracellularis*. As the fecal samples were positive by PCR, the rabbit was suspected of being infected with *L. intracellularis*. Thickening of the gut wall was also observed. To confirm the infection of *L. intracellularis*, we cultivated *L. intracellularis* by cell culture method [8]. Frozen (–80°C) intestine was homogenized and was used as the source material. Rat small intestinal cells (IEC-18; ATCC CRL 1589) 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 were centrifuged at 700 × g before transfer to an incubator. The incubator was kept at an atmosphere of 8.0% O_2 and 8.8% CO_2 at 37°C. The culture was incubated for 6 hr and the medium was replaced with DMEM containing gentamicin (30 μg/m*l*), and then was transferred to the incubator. At 6 days after infection, the infected monolayer examined by phase microscopy showed little morphological change and several detached cells (Fig. 1B). To detect intracellular *L. intracellularis*, we prepared rabbit antiserum against synthetic peptides in the *Lawsonia* surface antigen (LsaA) [9], which were amino acid residues from 41 to 56 in LsaA, and then *L. intracellularis* were detected by immunohistochemistry. These amino acid residues have no homology for other proteins and have antigenic epitope, and were synthesized by SIGMA Genosys Japan. Infected cells were fixed with 4% paraformaldehyde and then were permeabilized in 0.1% Triton X-100. Samples were stained with the antiserum for 1 hr at 37°C. After washing three times for 5 min in phosphate-buffered saline (PBS), samples were stained simultaneously with FITC labeled anti-rabbit IgG. Samples were placed in mounting medium and were visualized by fluorescence microscopy. Many intracellular bacteria reacted with anti-LsaA serum (Fig. 1A). Staining of the ileum section from the infected rabbit with a Dako EnVision System by anti-LsaA serum also showed clusters of many bacteria in the apical cytoplasm of enterocytes (Fig. 1C). In contrast, staining of the ileum section from the uninfected rabbit by anti-LsaA serum did not show the positive reaction in enterocytes (data not shown). These results suggested that anti-LsaA serum should be useful to detect intracellular *L. intracellularis* specifically by immunohistochemistry.

We next investigated if these synthetic peptides of LsaA were useful as an antigen in enzyme-linked immunosorbent assay (ELISA) to test the infectivity of *L. intracellularis* by serum samples. To coat the synthetic peptides on immuno plates for ELISA, 50 μl of the synthetic peptides (10 μg/m*l*) was added to a 96-well Immuno plate (Nunc) and was left overnight at 4°C. Then, the wells were blocked by 0.5%

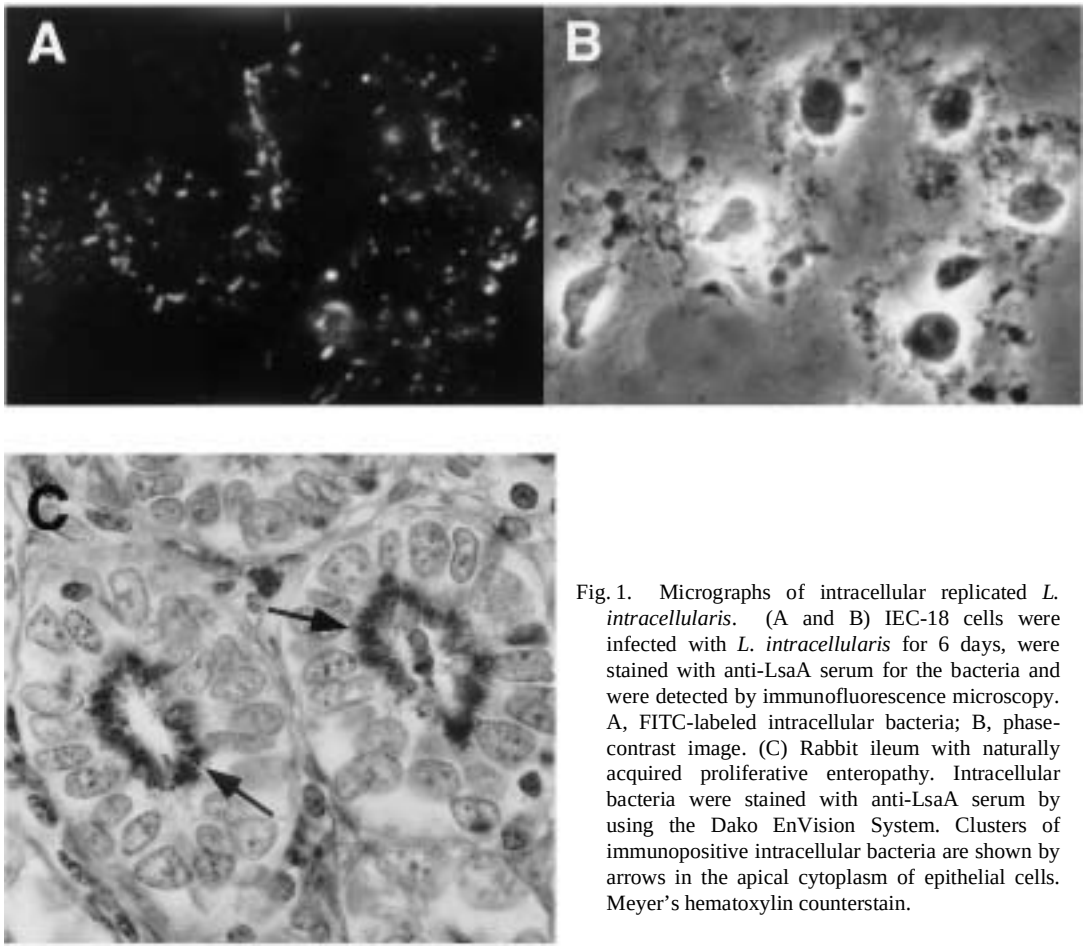

Fig. 1. Micrographs of intracellular replicated *L. intracellularis*. (A and B) IEC-18 cells were infected with *L. intracellularis* for 6 days, were stained with anti-LsaA serum for the bacteria and were detected by immunofluorescence microscopy. A, FITC-labeled intracellular bacteria; B, phase-contrast image. (C) Rabbit ileum with naturally acquired proliferative enteropathy. Intracellular bacteria were stained with anti-LsaA serum by using the Dako EnVision System. Clusters of immunopositive intracellular bacteria are shown by arrows in the apical cytoplasm of epithelial cells. Meyer's hematoxylin counterstain.

bovine serum albumin (BSA) for 30 min. Sera diluted 1/25 to 1/1,600 were added to the wells. The wells were incubated at 37°C for 1 hr, were washed, and then horseradish peroxidase-labeled protein G was added. The wells were incubated at 37°C for 1 hr, were washed, and substrate *o*-phenylenediamine was added. The absorbance was measured at 492 nm by an ELISA reader (model 450, Bio-Rad). ELISA showed that the serum from rabbit with naturally acquired *L. intracellularis* had a strong reaction with the synthetic peptides of LsaA (Fig. 2). In contrast, control serum (uninfected), which was confirmed PE negativity by PCR [6], did not show increase of OD_{492} values (Fig. 2). These results suggested that the ELISA with the synthetic peptides of LsaA would be useful for specific detection of *Lawsonia*-infected rabbits.

To further investigate the serological reactivity of the synthetic peptides of LsaA, sera from rabbits experimentally infected with *L. intracellularis* were tested by ELISA. Before infection, serum samples were collected from all rabbits and were tested by ELISA for a negative control. Fecal samples were collected and were tested for *L. intracellularis* DNA by PCR to assure PE negativity [6]. Three rabbits were inoculated with 5 m*l* of homogenized intestine from rabbit with naturally acquired *L. intracellularis* by a stomach tube. Fecal samples were collected from all rabbits every day for four weeks after inoculation and were tested by PCR [6]. The fecal samples were positive by PCR at around 10 days after inoculation. Serum samples were collected from the rabbits and were tested by ELISA with the synthetic peptides of LsaA. Diluted serum samples (1/25) from rabbit No. 2 showed a strong reaction and serum samples from rabbits No. 1 and 3 showed a comparatively stronger reaction than the control serum (Fig. 3). To check the ileum of rabbits No. 1 or 3 pathologically, the rabbits were killed at two or three weeks, respectively, and ileum sections were stained by the Dako EnVision System and antiserum. Although *L. intracellularis* were in the apical cytoplasm of enterocytes, they were comparatively fewer than samples from rabbits with naturally acquired PE or rabbit No. 2. Differences in the OD_{492} values of each rabbit may have been dependent on the condition of each rabbit. These results suggest that the synthetic peptides of LsaA might be useful as an antigen to detect for serum infected with *L. intracellularis* by ELISA.

Infection by *L. intracellularis* in domestic and experimental animals is becoming a more important infectious dis-

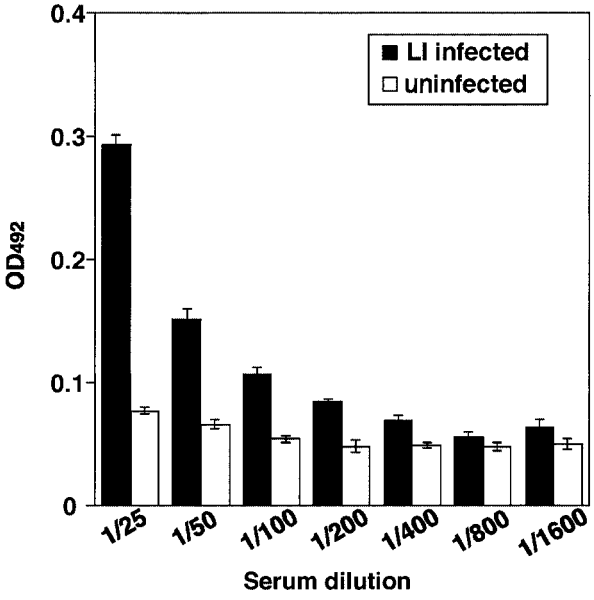

Fig. 2. ELISA absorbance of serum from naturally acquired proliferative enteropathy by using synthetic peptides of LsaA. Synthetic peptides were tested with rabbit serum infected or uninfected with *L. intracellularis* (LI). Values are averages and standard deviations of triplicate wells from three identical experiments.

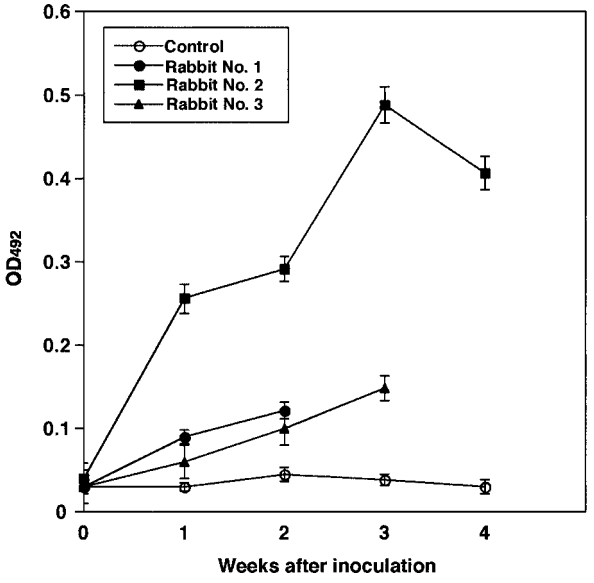

Fig. 3. ELISA absorbance of sera from rabbits experimentally infected with *L. intracellularis* by using synthetic peptides of LsaA. Synthetic peptides were reacted with three rabbit sera infected (rabbits No. 1, 2 and 3) or uninfected (Control) with *L. intracellularis*. Values are averages and standard deviations of triplicate wells from three identical experiments.

ease in Japan and is responsible for large economic losses. An easier and more rapid diagnosis method, which can deal with many samples at one time, should be established in Japan. Although we need accumulation of data by testing many samples, our data in this study will help to establish a serological method to diagnose PE.

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