NOTE *Bacteriology*

Detection of *Lawsonia intracellularis* Using Immunomagnetic Beads and ATP Bioluminescence

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ABSTRACT. *Lawsonia intracellularis* is an obligate intracellular pathogenic bacterium that causes proliferative enteropathy in various animals. The detection of *L. intracellularis* in clinical and environmental samples is necessary for the diagnosis of infection and epidemiological investigations. For the detection of *L. intracellularis* in fecal samples, we have developed an immunological method using immunomagnetic separation and ATP bioluminescence. Magnetic beads were coated with an anti-Lawsonia surface antigen (LsaA) antibody in order to capture the *L. intracellularis* in fecal samples from infected rabbits and the bacteria captured by the immunomagnetic beads were assayed by means of ATP bioluminescence. Our results showed that *L. intracelluraris* was detected by immunomagnetic separation of bacteria-holding magnetic beads and ATP-based bioluminescence, suggesting that our methods could be useful for the diagnosis of proliferative enteropathy.

KEY WORDS: immunomagnetic beads, *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]. The obligate intracellular bacterium, causative agent of PE, has been characterized as a novel genus and species and named *Lawsonia intracellularis* [8, 10]. *L. intracellularis* has been associated with colonization of enterocytes in rabbits, hamsters, rats, guinea pigs, swine, sheep, horses, white-tailed deer, dogs, arctic foxes, ferrets, and ostriches [1, 5, 13]. The development of *L. intracellularis* specific primers has made [6], its detection in feces by PCR possible, and serum IgG specific against *L. intracellularis* has been detected in pigs using an immunofluorescence antibody test [4, 7] and immunoperoxidase monolayer assay [2, 3]. However, techniques for the immunological detection of *L. intracellularis* had not been established. Previously, we reported on the detection of *L. intracellularis* by enzyme-linked immunosorbent assay with the *Lawsonia* surface antigen (LsaA) in rabbit PE [12], and in the present study, we describe a method detecting the bacterium in fecal samples using immunomagnetic separation and ATP-based bioluminescence, which is easier to perform and more rapid.

Immunomagnetic beads for *L. intracellularis* were made by immobilizing the anti-LsaA rabbit polyclonal antibody [9, 12] on protein A-coated paramagnetic beads ($6–7 \times 10^8$ beads/m*l*; New England BioLab) according to the manufacturer's instructions. Fecal samples from infected rabbits testing positive for PE by PCR (6) and uninfected rabbits, were homogenized (20% w/v) in phosphate buffered saline (PBS). Next, 20 μl of paramagnetic beads were added to 1 m*l* of each sample in 1.5-m*l* microcentrifuge tubes and mixed on a rotary mixer at room temperature for 30 min. The tubes were then placed in a magnetic particle concentrators (Dynal) for 5 min, gently inverted four times and left for 1 min. Carefully aspirating the supernatants, the recovery of paramagnetic beads was visually compared with control samples (1 m*l* of PBS from which 20 μl of beads were recovered). The intracellular ATP assay was performed using reagents from Toyo Ink and a Luminescencer-JNR AB-2100 (ATTO). The reagents included a luciferin-luciferase mixture and an ATP-releasing agent. All of the reagents were stored, prepared and used according to the manufacturer's recommendations. Fifty microliter samples of the magnetic beads with captured bacteria, were placed in the wells of a black 96-well microtiter plate and treated with 200 μl of ATP-releasing agent for 10 s at room temperature. The microtiter plate was then placed in a luminometer, and 100 μl of the luciferin-liciferase mixture was added. The resulting light emission was measured with a 10 s integration time and read as relative light unit. The ATP bioluminescence of the supernatants and washing solutions were also measured to check the reliability of the assay. The ATP concentrations were determined based on measurements for purified ATP supplied by the manufacturer.

We stained the immunomagnetic beads for DNA with DAPI and then took fluorescence photomicrographs to examine the status of capture of *L. intracellularis* by the beads. This showed that several bacteria may be captured by one bead and bead/bacteria clusters were formed (Fig. 1B). Very few bacteria may be captured nonspecifically (Fig. 1A). Since the number of captured bacteria could not be counted by fluorescence microscopy, a method that can provide a signal proportional to the cell numbers was needed. Thus, ATP bioluminescence was assessed for its ability to determine numbers of captured bacteria. *L. intracellularis* cannot be cultured using bacteriological media. As the colony forming units (CFU) for the captured *L. intra-*

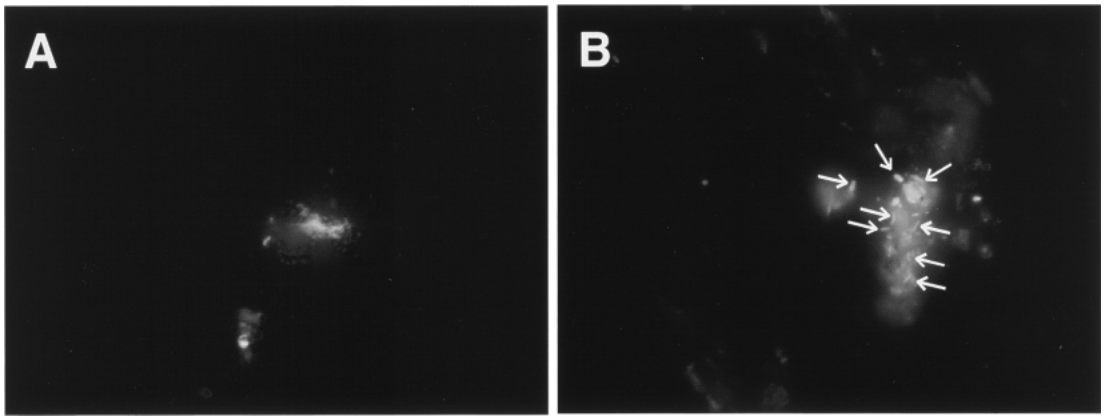

Fig. 1. Fluorescence micrographs of *L. intracellularis* captured by immunomagnetic beads. Bacteria in fecal samples from infected rabbits captured by normal rabbit IgG coated magnetic beads (A) and anti-LsaA antibody coated magnetic beads (B) were stained for DNA with DAPI and assayed by fluorescence microscopy. Arrows show bacteria.

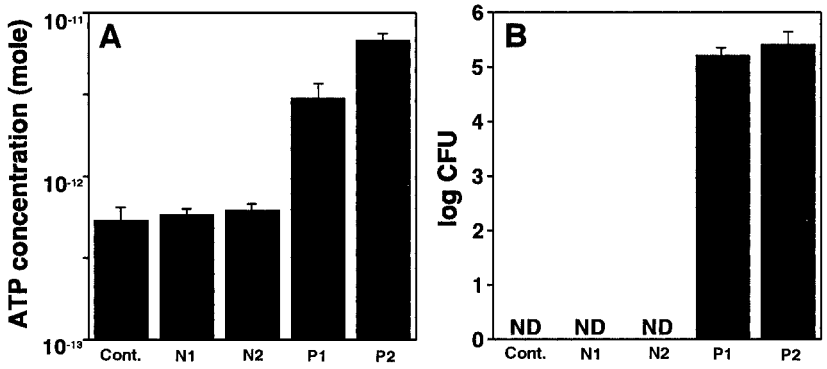

Fig. 2. Detection of *L. intracellularis* by ATP bioluminescence. (A) ATP concentrations of bacteria captured by anti-LsaA antibody coated magnetic beads from fecal samples of infected rabbits (P1 and P2) and uninfected rabbits (N1 and N2), and bacteria captured by normal rabbit IgG coated magnetic beads from fecal samples of infected rabbits (Cont.) were measured. Values are averages and standard deviations of triplicate wells from two identical experiments. (B) CFU of bacteria captured by anti-LsaA antibody coated magnetic beads from fecal samples of infected rabbits (P1 and P2) and uninfected rabbits (N1 and N2), and bacteria captured by normal rabbit IgG coated magnetic beads from fecal samples of infected rabbits (Cont.) were estimated through a comparison with data for *E. coli*. Values are averages and standard deviations of triplicate samples from two identical experiments. ND means not detectable.

cellularis could not be determined accurately, we estimated values for CFU through a comparison of our CFU data and ATP concentrations in *Escherichia coli* DH5α. CFU of the captured bacteria was calculated from the standard curve with *E. coli*. ATP concentrations were higher for anti-LsaA antibody coated magnetic beads exposed to fecal samples of infected rabbits than those exposed to fecal samples from uninfected rabbits or those of normal rabbit IgG coated magnetic beads exposed to fecal samples of infected rabbits (Fig. 2A). The CFU was estimated through a comparison with *E. coli* data. Our results indicate that about 2–3 × 10^5 CFU were captured by anti-LsaA antibody coated magnetic beads. However, CFU estimates could not be made for bacteria captured by anti-LsaA antibody coated magnetic beads exposed to fecal samples from uninfected rabbits or normal rabbit IgG coated magnetic beads exposed to fecal samples from infected rabbits since CFU were under the limits of detection (<10^3 CFU) (Fig. 2B). This suggests that anti-LsaA antibody coated magnetic beads detect *L. intracellularis* specifically.

PE caused by *L. intracellularis* infection in various animals is becoming a serious disease in Japan and is responsible for large economic losses. For this reason a more convenient and rapid diagnosis method, which can deal with many samples at one time, needs to be established. We need to collect much more data by testing many samples, but the

results of the present study will be very helpful in establishing an effective immunological method for diagnosing PE.

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