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Detection of *Lawsonia intracellularis* in diagnostic specimens by one-step PCR

Dong-Kyun Suh[1], Suk-Kyung Lym[2], You-Chan Bae[2], Keun-Woo Lee[3], Won-Pil Choi[3], Jae-Chan Song[3]*

[1]*Research Institute of Public Health and Environment, Taegu 706-100, Korea*
[2]*National Veterinary Research and Quarantine Service, Anyang 430-016, Korea*
[3]*College of Veterinary Medicine, Kyungpook National University, Taegu 702-701, Korea*

***Lawsonia intracellularis* is not culturable with a standard bacteriologic culture. One step PCR assay as a clinical diagnostic method was developed for the rapid detection of porcine proliferative enteritis (PPE) caused by *L. intracellularis*. Primers were designed based on the *p78* DNA clone of *L. intracellularis*. The one step PCR resulted in the formation of a specific 210-bp DNA product derived from *L. intracellularis*. The nonspecific amplification product was not detected with swine genomic DNA or other bacterial strains causing similar symptoms to *L. intracellularis* infection. The one step PCR was as sensitive as 100 pg of *L. intracellularis* genomic DNA. We applied this method to field specimens diagnosed as PPE by macroscopic observation. Of 17 mucosal scraping specimens, 16 (94%) were identified as positive to PPE and 15 (88%) of 17 feces specimens. These results suggest that the one step PCR can be used as a rapid diagnostic method for *L. intracellularis* infection.**

Key word: *Lawsonia intracellularis*, porcine proliferative enteritis, diagnosis, PCR.

Introduction

Porcine proliferative enteritis (PPE), known as ileitis, intestinal adenomatosis, or necrotic enteritis, is a naturally occurring disease that can affect pigs from their weaning to young adult stage. PPE was formerly known to be caused by *Camplyobacter*-like organism or ileal symbiont intracellularis [5, 15]. A recent work, however, have established that the causative agent was *L. intracellularis*, an obligate intracellular bacterium [19]. The disease is of economic importance due to death loss, increased medication costs, poor weight gain, and decreased feed conversion, etc. Estimates of the reductions in the weight gain and feed conversion efficiency were generally 20 to 30% [7, 17]. Various treatment programs to control the clinical signs of PPE were hampered by lack of data on the causative agent, antimicrobial susceptibility, and likely host responses to treatment. A common practice is to apply antimicrobials to affected pigs. However, antibiograms on a limited number of isolates are now available [13, 21].

A key element to rational therapy and effective control of the diseases is a rapid and accurate identification of etiologic agents. PPE is diagnosed by observation of gross lesions and is confirmed by observation of typical histopathological lesions in which the intracellular curved rods is demonstrated by special staining methods [8, 18]. The final decision should be made through the isolation of the causative agent. However, the isolation and culture of this organism require specialized cell culture techniques [11, 14, 20]. Recently, polymerase chain reaction (PCR) techniques have been successfully used to detect the DNA derived from the causative agent in specimen on swabs of intestine [4, 24]. The detection of the causative agent by PCR method is more sensitive in the detection of *L. intracellularis* than either fluorescent antibody (FA) staining or conventional histopathological techniques [2, 3]. The sensitivity of PCR for the detection of L. intracellularis was evaluated in the previous reports [3, 9, 10]. These reports have been particularly focused on nested PCR to detect the specific DNA from causative agent, because unknown inhibitory factors which can decrease the sensitivity and specificity might be contaminated during the preparation of template DNA. The nested PCR method increases the sensitivity to additional 10 to 100-fold [3]. Though the nested PCR is a sensitive method in the detection of *L. intracellularis*, it is time consuming and laborious. Therefore, a more convenient PCR method should be developed. In this study, we developed a sensitive PCR-based assay for the detection of *L. intracellularis* in field specimen without reamplification step of PCR products.

*Corresponding author
Phone: 82-53-950-5961; Fax: 82-53-950-5955;
E-mail: songjach@kyungpook.ac.kr

Materials and Methods

Preparation of template DNA

Bacterial strains used in this study were *Lawsonia intracellularis*, *Salmonella typhimurium (B)*, *S. enteritidis (D)*, *S. cholerasuis (C)*, *Serpulina hyodysenteriae (B204)*, *Campylobacter jejuni*, *Listeria monocytogenes*, *and Escherichia coli (ML1410)*. All these strains were obtained from National Veterinary Research and Quarantine Services, Korea. To determine the specificity of PCR primers synthesized for reference strains, bacterial DNA was extracted as previously described [23]. DNA from mucosal scrapings of swine intestinal specimens diagnosed as PPE was extracted by the method described by Jones et al [3]. The ileal mucosa from pigs with PPE was scraped from the ileum and homogenized using a tissue grinder. The homogenate was centrifuged at 750 × g for 10 min at room temperature, and the supernatant was filtered sequentially through 5-μm, 1.2-μm, and 0.8-μm filters. The filtrate was centrifuged at 8,000 × g for 10 min. After discarding the supernatant, the pellet was resuspended in phosphate buffered saline (PBS) and it was referred to as an infected mucosal filtrate. 50 μl of 20% diatomaceous earth (DE) suspension in 0.17 M HCl was vortexed with 50 μl of the infected mucosal filtrate in a sterile microcentrifuge tube containing 950 μl of lysis buffer consisting of 5 M guanidine thiocyanate (GuSCN), 22 mM EDTA, 0.05 M Tris.Cl (pH 6.4), and 0.65% Triton X-100. The specimen was allowed to stand at room temperature for 10 min, vortexed, and then centrifuged at 14,000 × g for 20 sec. The lysis buffer was drawn off with a pipette. The pellet was dried at 56°C for 15 min and dissolved in TE buffer. After centrifugation at 12,000 × g for 2 min, the supernatant was stored at -20°C. Fecal specimen (0.2 g) was suspended in lysis buffer. The suspension was vortexed and then centrifuged at 14,000 × g for 20 sec after standing for 1 h at room temperature. The supernatant was placed in a tube containing 50 μl of DE suspension. The further processing was performed with the same procedure as described above for the extraction of DNA from the mucosal filtrate.

Primers and Polymerase chain reaction

A sequence specific for *L. intracellularis* (GenBank accession number L08049) was used to construct PCR primers. Two primers of 23 nucleotides in length were synthesized with a DNA synthesizer (Bioneer Co. Cheongju, Korea) as follows: forward primer 5'-GCAGCACTTGCAAACAATAAACT-3'; reverse primer 5'-TTCTCCTTTCTCATGTCCCATAA-3'. The two primers corresponded to nucleotides 110 to 132 and 297 to 319, respectively, and defined a 210-bp DNA fragment on PCR reaction. PCR mixture (50 μl) contained 5 μl of 10 × PCR buffer, 3 μl of 25 mM $MgCl_2$, 4 μl of 10 mM deoxynucleotide triphosphate mixture, 20 pmol of each primers, 1 μl of DNA template, and 0.5 unit of Taq Polymerase (Takara Co. Japan). PCR reaction was performed using an automatized thermal cycler (Robocycler, Stratagene, U.S.A). The initial mixture was heated at 94°C for 5 min. This step was followed by 45 cycles, each step consisting of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and polymerization at 72 °C for 1 min, followed by additional polymerization at 72 °C for 5 min. Electrophoresis was performed on 5 μl of the PCR product in a 1.8% metaphore agarose gel with Tris acetate electphoresis buffer (TAE, 0.04 M Tris, 0.001 M EDTA, pH 7.8). The EtBr-stained agarose gels were photographed under an UV transilluminator, and the DNA band pattern was analysed using an Eagle Eye II (Stratagene, U.S.A) according to the manufacturer's manual.

Cloning and sequencing of PCR product

PCR product was purified using a GeneClean II kit (Invitrogen, Carlsland, CA) after agarose gel electrophoresis and then cloned into *pBluescript* KS plasmid in *EcoRV* site. The 10 cloned PCR products were sequenced by the PCR sequencing method using a Top™ DNA sequencing kit (Injae Co. Cheongju, Korea). The sequence of the products were identified by comparison with the previous report [6] obtained from the GenBank.

Results

Specificity and sensitivity of PCR amplification

DNA isolated from the intestinal specimens of pigs with PPE as well as DNA from several other bacterial strains,

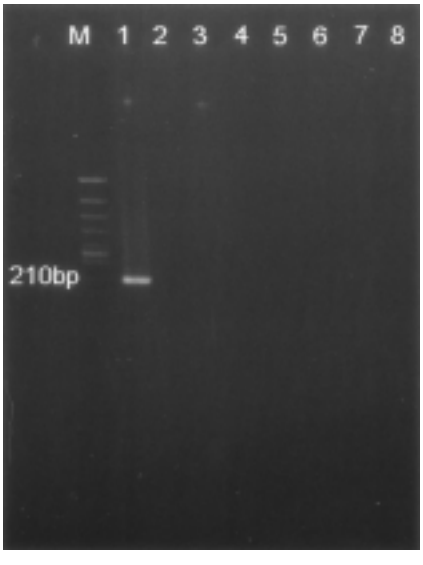

Fig 1. Specificity of one-step PCR assay for the detection of *L. intracellularis* genomic DNA with 45 cycles. M: *φX174* digested by **Hae** III; Lane 1: *L. intracellularis* genomic DNA; Lane 2 : *S. typhimurium*; Lane 3 : *S. enteritidis*; Lane 4 : *S. cholerasuis*; Lane 5 : *S. hyodysenteriae*; Lane 6 : C. *jejuni*; Lane 7 : *L. monocytogenes*; Lane 8 : E. *coli* (ML1410).

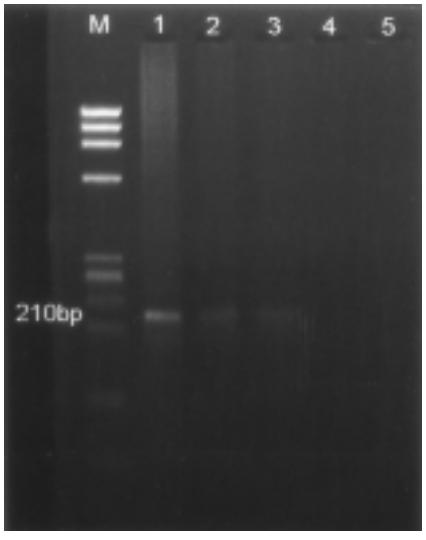

Fig 2. PCR amplification patterns of various amounts of *L. intracellularis* genomic DNA. Amplification was performed by 35 cycles.

M : *φX174* digested by *Hae* III; Lane 1 : 100 ng of template DNA; Lane 2 : 10 ng of template; Lane 3 : 1 ng of template; Lane 4 : 100 pg of template; Lane 5 : 10 pg of template.

including *S. hyodysenteriae*, *Campylobacter spp*., and *Salmonella spp*. which cause intestinal diseases in swine, were used as templates in PCR reaction. The primer designed for this study produced the expected DNA fragment in the PCR reaction with template DNA purified from the intestinal specimens of pigs with PPE, but did not produce any nonspecific amplified DNA fragments with swine geno- mic DNA or other bacterial strains (Fig. 1).

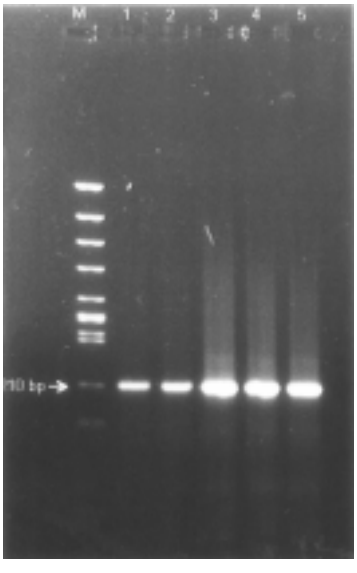

Fig 3. PCR amplification patterns of various numbers of cycles. 100 pg of template DNA was amplified.

M : *φX174* digested by Hae III; Lane 1 : 35 cycles; Lane 2 : 40 cycles; Lane 3 : 45 cycles; Lane 4 : 50 cycles; Lane 5 : 55 cycles.

Various amounts of *L. intracellularis* genomic DNA were prepared by 10-fold serial dilutions from 1 μg to 100 pg and subjected to PCR reaction to determine the sensitivity of the PCR assay. The detection limit of the PCR with 35 cycles was in the range up to 1 ng of template DNA (Fig. 2). To increase the sensitivity of the PCR assay, the template DNA diluted by a 10-fold serial dilution from 100 ng to 10 pg was amplified with 40, 45, 50 and 55 cycles of the PCR reactions. The best amplification condition of PCR system was 100 pg of the template DNA and 45 cycles in the PCR reaction. There was no increase in the sensitivity with 50 and 55 cycles of amplification (Fig. 3).

Analysis of clinical field strain

Seventeen pigs diagnosed as PPE by macroscopic examination were used to determine the accuracy of the PCR assay for the detection of *L. intracellularis* infection (Table 1). Mucosal scraping and fecal samples were obtained from each pig. Of 17 mucosal scrapings, 16 specimens produced the specific amplified DNA fragment by the PCR assay. Of 17 fecal samples, 15 specimens were positive by the PCR assay. No amplified DNA was detected both in the mucosal and fecal specimens from one pig. Whereas 13 intestinal specimens were positive by conventional diagnostic methods (Table 1). Microscopically, numerous intracellular, curved, rod-shaped

Table 1. Comparison of PCR assay with conventional methods for the detection of PPE caused by *L. intracellularis* from field samples

No. of pigs	Macroscopic examination	Conventional methods*	PCR assay	
			Mucosal scrapings	feces
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	-	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	-	+	-
11	+	-	-	-
12	+	+	+	+
13	+	+	+	+
14	+	+	+	+
15	+	+	+	+
16	+	-	+	+
17	+	+	+	+
Total	17	13	16	15

*Conventional methods include H & E staining and silver staining of the intestinal sections.

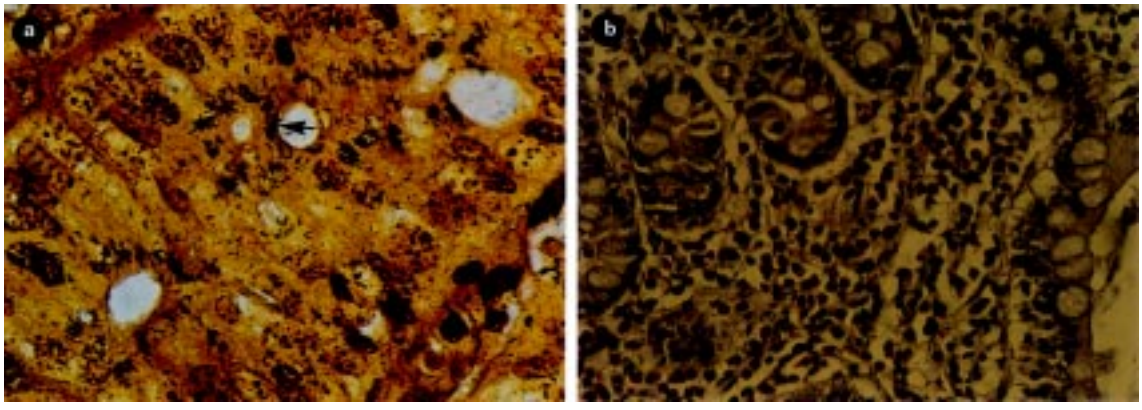

Fig 4. Section of the ileum from a pig infected with *L. intracellularis*.

a : Presence of numerous intracellular, curved, rod-shaped organisms (arrow) in the apical cytoplasm of the crypt epithelial cells, Warthin-Starry staining, × 1,000.

b : The crypts are filled with inflammatory cells, H&E staining, × 400.

organisms were found in the apical cytoplasm of the crypt epithelial cells in the ileum of a pig infected with *L. intracellularis* (Fig. 4a). In addition, the crypts were filled with inflammatory cells (Fig. 4b).

Discussions

Porcine proliferative enteropathy (PPE) is a common enteric disease affecting growing pigs raised under various management systems around the world [15]. The causative agent has been recently classified as a new genus and species of the class proteobacteria named *L. intracellularis* [19]. Farm prevalence studies in several countries including Europe, Asia, and North America indicated that 24 to 47% of pig farms showed a serious incidence with ileitis in the past several years [1, 12 22]. Diagnosis of *L. intracelluaris* infection primarily depends on the observation of gross and histopathological lesions in which the intracellular curved rods are demonstrated by silver stains because the isolation and cultivation of this obligate organism require specialized cell culture techniques [8, 18, 20]. Recently, PCR/ Southern hybridization, and nested PCR assays for the detection of *L. intracellularis*-specific DNA have been reported to be more sensitive than other conventional methods [3, 10]. However, the particular nested PCR assay to confirm the amplified PCR products is laborious and time-consuming.

To minimize this problem, we reconstructed the previously reported PCR analysis system [10] which included synthesis of DNA primers, annealing temperature and number of reaction cycles. In the present study, we performed an one step PCR assay to detect a *L. intracellularis*-specific DNA without reamplification step of a PCR product. The PCR product was corresponded to the predicted molecular weight of the DNA fragment. In addition, the sequences of 210-bp PCR product were identical to the source DNA sequences. Nonspecific amplification product was not detected with untargeted bacterial DNA which could be normally present in the porcine intestine and feces including swine genomic DNA. The number of amplification cycles was one of the important factors for increasing the sensitivity of the PCR assay. The increase in the number of amplification cycles may produce nonspecific DNA. But nonspecific DNA was not detected in this study in spite of increasing the amplification cycles from 45 to 55 cycles. It is necessary that the amount of template DNA should be used as little as possible. Forty five cycles of the PCR reaction with 100 pg of template DNA for increasing the sensitivity of detection did not affect the specificity of amplification result. The increased sensitivity of our PCR protocol over the previous report [16] (about 10 times) was likely due to the increase in the number of PCR cycle.

In this study, the accuracy for the detection of *L. intracellularis* by the PCR assay was 94.1% (16/17) with the intestinal mucosal samples, 88.2% (15/17) with the fecal samples, but only 76.4% (13/17) by the conventional examination based on the microscopic observation. These results indicated that the one step amplification by PCR reaction was more sensitive for the detection of *L. intracellularis* than the conventional method. One fecal specimen was negative by the PCR analysis, but the respective mucosal specimen was positive. The result might be explained due to the sensitivity differences between sources, storage of feces at -80°C, subsequent DNA extraction, and PCR amplification [3]. It had been reported that the PCR assay could detect 10^3~10^4 *L. intracellularis* organisms/g of feces and 10^1 organisms/mucus [3]. We applied this method to the slaughter pigs with thick ileum and mesentery for analysing the accuracy of macroscopic ability to detect pig with PPE and the availability of the PCR assay for screening the prevalence of *L. intracellularis* infection in the pig farms. Macroscopic

examination for the thick intestines of slaughter pigs was sensitive, but not specific for detecting pigs with PPE. Jones *et al* [10] reported that the age of onset of clinical signs was a critical determinant of detecting lesions at slaughter. The low specificity might be attributable to the age of pigs with PPE, ranging 6~20 weeks old.

In conclusion, the development of an one step PCR assay for the detection of *L. intracellularis* may not only facilitate a rapid clinical diagnosis of PPE but also enable epidemiological studies and screening to prevent clean herds from the transmission of *L. intracellularis* by animal movement.

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