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

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*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 *Campylobacter*-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.
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’-GCAGCACTTGCAAACAT-3’; reverse primer 5’-TTCTCCTTTTCTCATGTC-CCATAA-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 MgCl2, 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% metaphere agarose gel with Tris acetate electrophoresis 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 TopTM 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).