

Simultaneous detection of *Lawsonia intracellularis*, *Brachyspira hyodysenteriae* and *Salmonella* spp. in swine intestinal specimens by multiplex polymerase chain reaction

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A multiplex PCR assay was developed for the simultaneous detection of the etiologic agents associated with porcine proliferative enteropathies (PPE), swine dysentery (SD) and porcine salmonellosis (PS) in a single reaction using DNA from swine intestinal samples. Single and multiplex PCR amplification of DNA from *Lawsonia intracellularis*, *Salmonella typhimurium* and *Brachyspira hyodysenteriae* with each primer set produced fragments of the predicted size without any nonspecific amplification, 210-bp, 298-bp and 403-bp bands, respectively. The single PCR assay could detect as little as 100 pg of purified DNA of *S. typhimurium* and *L. intracellularis*, and 50 pg of *B. hyodysenteriae*, respectively. However, multiplex PCR turned out to be 10 times lower sensitivity with *S. typhimurium* compared with single PCR. With 23 swine intestinal specimens suspected of having PPE, SD and/or PS, the multiplex PCR assay showed identical results with conventional methods except one. In conclusion, this multiplex PCR is a feasible alternative to standard diagnostic methods for detection of *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. from swine intestinal specimens.

Key words: *Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, Multiplex PCR, *Salmonella* spp.

Introduction

Porcine proliferative enteropathies (PPE) caused by *Lawsonia intracellularis*, swine dysentery (SD) by *Brachyspira hyodysenteriae* and porcine salmonellosis (PS) are acknowledged as important diseases of suboptimal performance and mortality in grower-finisher pigs, causing tremendous financial loss due to death of pigs, decreased rate of growth and poor feed conversion [22]. PPE, also

known as ileitis, intestinal adenomatosis, or necrotic enteritis is a naturally occurring disease that can affect pigs from their weaning to young adult stage. The disease is of economic importance due to death loss, increased medication costs, poor weight gain and decreased feed conversion, etc. Estimates of the reduction in the weight gain and feed conversion efficiency were 20 to 30% [14,21]. Salmonellosis is a worldwide problem and causes zoonotic disease. PS manifests itself in postweaning pigs of all ages, and is most often attributed to *S. choleraesuis* var. *kunzendorf* and *S. typhimurium*. Infection in swine typically results in diarrhea with septicemia and pneumonia more common in older swine. Prevalence of *Salmonella* infection is widespread from 3 to 21% depending in part on which tissues were examined [24]. Diarrhea is the most consistent sign of SD. As the diarrhea progresses, watery stools containing blood, mucus and shreds of white mucofibrinous exudate are seen, with concurrent staining of rear quarters [12]. SD also causes a tremendous financial loss due to the expenses for therapy because it seems to occur in a cyclic manner with 3 to 4 weeks intervals.

Diagnosis of PPE had been done by the observation of typical histopathological lesions characterized by the marked proliferation of immature enterocytes within crypts of intestine; affected cells were demonstrated by Warthin-Starry (silver) stains. However, the culture and isolation of this organism require specialized cell culture techniques [16,17,19]. Culture was also the most widely used tool for *Salmonella* detection. However, the current standard laboratory procedure to culture and identify *Salmonella* spp. takes approximately 4 to 7 days [8]. In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small numbers of organism. The diagnosis of SD is based on herd history, clinical signs, observation of characteristic intestinal lesions and isolation of *B. hyodysenteriae* from feces or the intestine. Isolating *B. hyodysenteriae* from other intestinal bacteria becomes more difficult when attempting to recover the organism from swine infected with *B. hyodysenteriae* but having chronic

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diarrhea or no diarrhea. Laboratory confirmation of *B. hyodysenteriae* by culture is based upon colony morphology, pattern and intensity of hemolysis and other growth characteristics, all of which are very similar for nonpathogenic *B. innocens*. As a result, a definitive diagnosis of swine dysentery can be very challenging [13].

The advent of molecular techniques has allowed for the development of more rapid diagnostic test of pathogenic organism. Use of polymerase chain reaction (PCR) has been reported for the definitive identification of several pathogenic organisms mentioned above [4-7,15]. We have already developed one step PCR for detection of *L. intracellularis* from diagnostic sample [28]. This study further developed a multiplex PCR assay for simultaneous detection of the etiologic agents associated with PPE, SD and PS to reduce the preparation and analysis time required to identify multiple target sites in 1 assay. Also, we tested whether this assay can be a useful alternative to single PCR and used as a complement test for standard diagnostic method.

Materials and Methods

Bacterial strains and DNA

L. intracellularis genomic DNA, 11 *Salmonella* spp., 10 *Brachyspira* spp. including 8 field isolates and 3 other enteric bacteria were used in this study (Table 1). All bacterial strains were identified biochemically and serologically [3]. Chromosomal DNA of *Salmonella* spp. and other bacterial strains listed in Table 1 were isolated as previously described [1,23].

Preparation of DNA from intestinal specimens

A total of 23 porcine intestinal specimens consisting of feces and mucosal scrapings were obtained from the slaughter pigs and field cases of Youngnam province during the periods between 1997 and 2000. Culture and identification of *Salmonella* spp. and *B. hyodysenteriae*, and histopathological examinations of tissue specimens for *L.*

intracellularis were performed by standard techniques [3,18]. DNA from mucosal scrapings of swine intestinal specimens diagnosed as PPE was extracted by the method described by Jones *et al.* [2,17]. The ileal mucosa from pigs with PPE was scraped from the ileum and homogenized in tissue grinder. The homogenate was centrifuged, and the supernatant was filtered sequentially through 5 µm, 1.2 µm and 0.8 µm filters. A 20% diatomaceous earth suspension (50 µl) in 0.17 M HCl was vortexed with infected mucosal filtrate (50 µl) 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 lysis buffer was drawn off with a pipette, 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, vortexed and was then centrifuged at 14,000 × g for 20 sec after standing for 1 hr at room temperature. The supernatant was placed in a tube containing 50 µl of DE suspension. Further processing needed was the same as described above for the extraction of DNA from mucosal filtrate.

Oligonucleotides and PCR reaction

The primers for specific amplification of *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. by multiplex PCR assays were designed from Bioneer Co. (Korea) (Table 2). The 50 µl of PCR mixture 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 each DNA template and 0.5 unit of *Taq* DNA polymerase (Takara, Japan). PCR amplification was conducted on a DNA thermocycler (Robocycler; Stratagene, USA). The initial mixture was heated to 94°C for 5 min. This step was followed by 45 cycles, each 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. The presence of PCR

Table 1. List of bacterial strains (DNA) and sources

Bacterial strains	Sources	Bacterial strains	Sources
<i>L. intracellularis</i> DNA	NVRQS*	<i>B. innocens</i>	ATCC29796
<i>S. enteritidis</i> (D1)	ATCC13076	<i>B. hyodysenteriae</i> (B204)	ATCC31287
<i>S. reading</i> (B)	ATCC11511	<i>B. hyodysenteriae</i> 1	swine
<i>S. durazzo</i> (A)	ATCC6967	<i>B. hyodysenteriae</i> 2	swine
<i>S. typhimurium</i> (B)	ATCC29946	<i>B. hyodysenteriae</i> 3	swine
<i>S. typhi</i> (D)	NVRQS	<i>B. hyodysenteriae</i> 4	swine
<i>S. newport</i> (C2)	NVRQS	<i>B. hyodysenteriae</i> 5	swine
<i>S. derby</i> (B)	NVRQS	<i>B. hyodysenteriae</i> 6	swine
<i>S. muenchen</i> (C2)	NVRQS (chicken)	<i>B. hyodysenteriae</i> 7	swine
<i>S. montevideo</i> (C1)	NVRQS (chicken)	<i>B. hyodysenteriae</i> 8	swine
<i>S. choleraesuis</i> (C1)	NVRQS (swine)	<i>Escherichia coli</i> (ML1410)	NVRQS
<i>S. meleagridis</i> (E1)	NVRQS (chicken)	<i>Campylobacter jejuni</i>	ATCC33560
		<i>Listeria monocytogens</i>	ATCC15313

*NVRQS: National Veterinary Research and Quarantine Services, Ministry of Agriculture and Forestry, Korea

Table 2. Primers for multiplex PCR amplification of *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. from porcine intestinal specimens

Organism	Name	Sequences (forward; reverse)	Size
<i>L. intracellularis</i>	LIR	5'-GCAGCACTTGCAAACAATAAACT-3'	210 bp
	LIR	5'-TTCTCCTTTCTCATGTCCATAA-3'	
<i>B. hyodysenteriae</i>	BHF	5'-GCTGGAGATGATGCTTCTGG-3'	403 bp
	BHR	5'-GTCCAAGAGCTTGGCTGTTC-3'	
<i>Salmonella</i> spp.	SAF	5'-TTGGTGTATTATGGGGTCGTT-3'	298 bp
	SAR	5'-GGGCATACCATCCAGAGAAA-3'	

products was determined by electrophoresis of 5 ml of the amplified products in a 1.8% metaphore agarose gel with tris boric acid electrophoresis buffer (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA, pH 7.8) and visualized using the Eagle Eye II (Stratagene, USA) according to manufacturer's manual.

Sensitivity and specificity of multiplex PCR

To assess the minimal amount of DNA detectable by multiplex PCR, genomic DNA from *L. intracellularis*, *B. hyodysenteriae* B204 and *S. typhimurium* ATCC 29946 were prepared by 10-fold serial dilutions from 100 ng to 10 pg and subjected to PCR reaction. For specificity determination, DNA from all strains of bacteria listed in Table 1 was used in DNA amplification. Negative control for clinical samples were collected from slaughter pigs of the farm from which there were no previous symptoms or history of PPE, SD and PS for last years since 1998. They were determined whether the 3 organisms were detected by the bacteriological cultures for *Salmonella* spp. and *B. hyodysenteriae*, and by the histological examination for *L. intracellularis*.

Cloning and sequencing of PCR product

To confirm the identity of the PCR products, they were purified by using GeneClean II kit (Invitrogen, USA) after agarose gel electrophoresis and then cloned into pBluescript KS plasmid in *EcoRV* site. The 8 cloned each PCR products were sequenced by PCR sequencing method with TopTM DNA sequencing kit (Injae, Korea). The identities of the products were confirmed by comparison of the sequence with previous report obtained from the GenBank [9-11].

Results

PCR reaction

Single PCR amplification of purified DNA from *L. intracellularis*, *S. typhimurium* and *B. hyodysenteriae* with each primer set resulted in a fragment of the predicted size: 210-bp, 298-bp and 403-bp bands, respectively (Fig. 1). Also, multiplex PCR amplification of purified DNA from each species yielded products corresponding to the same molecular weight of DNA as single PCR. To determine the

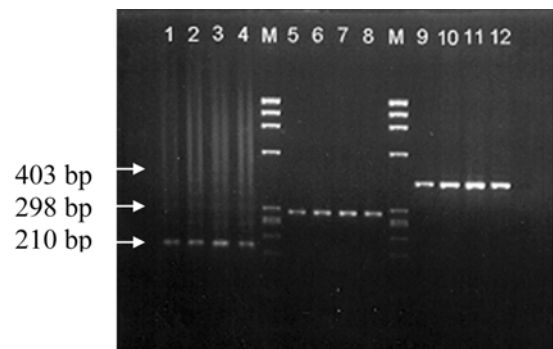


Fig. 1. Single PCR amplified DNA pattern of *L. intracellularis*, *S. typhimurium* and *B. hyodysenteriae* with 45 cycles at different annealing temperature. M; ϕ X174 digested by *Hae* III, Lane 1~4; *L. intracellularis* template DNA at 52°C, 54°C, 56°C and 58°C, respectively, Lane 5~8; *S. typhimurium* template DNA at different DNA at 52°C, 54°C, 56°C and 58°C, respectively, Lane 9~12; *B. hyodysenteriae* template DNA at 52°C, 54°C, 56°C and 58°C, respectively.

minimal detectable concentration of each template DNA, PCR was conducted on serial dilutions of each purified DNA from 100 ng to 10 pg. The single PCR assay could detect as little as 100 pg of purified DNA for *S. typhimurium* and *L. intracellularis*, and 50 pg for *B. hyodysenteriae*, respectively. However, multiplex PCR resulted in a sensitivity 10 times lower with *S. typhimurium* (Fig. 2). Subcloning of the amplified product from *L. intracellularis*, *S. typhimurium* ATCC 29946 and *B. hyodysenteriae* B204 into pBluescript KS strain and sequencing of the product indicated that it had the identical sequences with previous report obtained from the GenBank [9-11].

Specificity and sensitivity of multiplex PCR

In order to analyze the specificity of the PCR assay, DNA isolated from each bacterial strains as well as DNA from several other bacterial strains including *E. coli*, *Campylobacter* spp. and *Listeria monocytogenes* which cause intestinal diseases in swine, was used as a template DNA in each PCR reactions. The PCR assay produced the expected DNA fragment with template DNA purified each bacterial strains, and did not produce any nonspecific amplified DNA fragments derived from other untargeted organisms listed in Table 1 (Figs. 3 and 4).

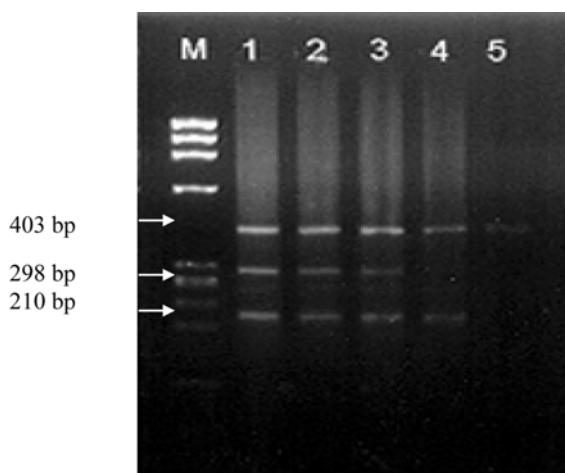


Fig. 2. Sensitivity of the amplified DNA from *L. intracellularis*, *B. hyodysenteriae*, and *S. typhimurium* by multiplex PCR. Each template DNA was serially diluted. M; ϕ X174 digested by *Hae* III, Lane 1; 100 ng of template DNA, Lane 2; 10 ng of DNA, Lane 3; 1 ng of DNA, Lane 4; 100 pg of DNA, Lane 5; 50 pg of DNA.

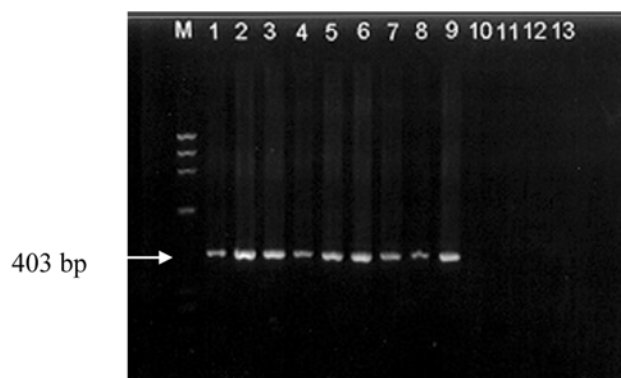


Fig. 3. Specificity of the multiplex PCR for detection of *B. hyodysenteriae* with 45 cycles. M; ϕ X174 digested by *Hae* III, Lane 1; *B. hyodysenteriae* ATCC31287, Lane 2-9; *B. hyodysenteriae* isolates, Lane 10; *B. innocens*, Lane 11; *Campylobacter jejuni*, Lane 12; *Escherichia coli*, Lane 13; *Listeria monocytogens*.

Evaluation of clinical samples by multiplex PCR

A total of 23 swine intestinal samples suspected of having PPE, SD and/or PS alone or in combinations were screened by cultivation and/or histopathological examination and multiplex PCR. Multiplex PCR assay yielded PCR products alone or in combination corresponding to the expected molecular weight of DNA from *L. intracellularis*, *Salmonella* spp. and *B. hyodysenteriae*: 210-bp, 298-bp and 403-bp bands, respectively (Fig. 5). Out of the 23 specimens, all specimens had multiplex PCR assay results that corresponded to the results of conventional method except one sample. It was negative with *L. intracellularis* by histopathological examination of tissues, but positive with multiplex PCR (Table 3).

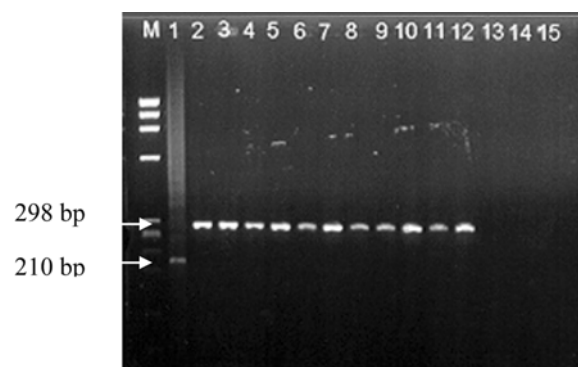


Fig. 4. Specificity of multiplex PCR for detection of *L. intracellularis* and *Salmonella* spp. M; ϕ X174 digested by *Hae* III, Lane 1; *L. intracellularis*, Lane 2-12; *Salmonella* spp. listed in Table 1., Lane 13; *Listeria monocytogenes*, LaLane 14; *Campylobacter jejuni*, Lane 15; *E. coli*.

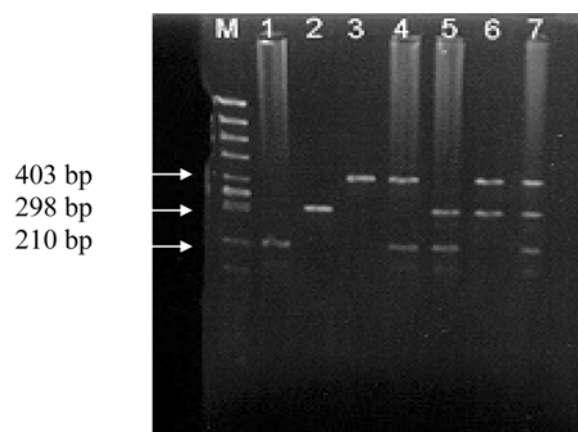


Fig. 5. Results of the multiplex PCR for detection of individual or combinations of bacterial agents from porcine intestinal feces. M; ϕ X174 digested by *Hae* III, Lane 1; *L. intracellularis* DNA, Lane 2; *S. typhimurium*, Lane 3; *B. hyodysenteriae*, Lane 4; *L. intracellularis* & *B. hyodysenteriae*, Lane 5; *L. intracellularis* & *S. typhimurium*, Lane 6; *S. typhimurium* & *B. hyodysenteriae*, Lane 7; *L. intracellularis* & *S. typhimurium* & *B. hyodysenteriae* (sample from herd No. 6).

Discussion

Recently, DNA sequences unique to each of the bacterial agents associated with PPE, SD and PS were independently identified. Moreover, DNA analysis techniques have been shown to be more sensitive than standard diagnostic methods for *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. [6,17,26]. In terms of each single PCR, PCR/Southern hybridization and PCR assay for the detection of *L. intracellularis* specific DNA have proven to be more sensitive than other conventional methods [7,17]. However, the PCR methods particularly focused on nested PCR to confirm the amplified PCR products which was laborious and time consuming. To minimize this problem, the present

Table 3. Results of conventional methods and multiplex PCR for detection of 3 organisms from porcine intestinal specimens

Organisms	No. positive	
	Conventional methods	Multiplex PCR
<i>L. intracellularis</i>	3	4(3) [†]
<i>Salmonella</i> spp.	10 [‡]	10
<i>B. hyodysenteriae</i>	4 [‡]	4
Negative	6	5
Total	23	23

[†]No. positive in feces[‡]Two samples were co-infected with *L. intracellularis*

study reconstructed the previously reported PCR analysis system, which included synthesis of DNA primers, annealing temperature and the number of reaction cycles. The one step PCR assay could detect a predicted 210-bp PCR product, which was identical to the source DNA sequences without the reamplification step of PCR product. Jones *et al.* [17] detect as few as 10¹ and 10³ *L. intracellularis* from 1 cm² of intestinal mucosa and one g of feces, respectively. However, a nonspecific band was detected from the PCR product amplified with fecal DNA. The numbers of amplification cycles are one of the important factors for increasing the sensitivity in PCR and might produce nonspecific DNA. However, nonspecific DNA was not detected in this study despite an increase in the amplification cycles from over 45 cycles. The increased sensitivity of this PCR protocol was about 10 times over that previously reported and likely due to the increase of the number of PCR cycles [20].

Sensitivity of the PCR for detection of *Salmonella* spp. was up to 100 pg of DNA. This was comparable to an earlier report in which 27 pg of purified chromosomal DNA were needed for detection of *Salmonella* spp. by PCR [25]. This sensitivity was lower than the detection limit of 330 fg by Nguyen *et al.* [23]. Earlier studies have described PCR-based probes for detection of *Salmonella* spp. [4,25,29]. There seems to a limitation of this PCR that it does not differentiate *Salmonella* spp. at the level of species because we used *Salmonella* common primer set from *invA* gene, which enable *Salmonella* spp. to invade the cell. Only a limited number of serotypes have been associated with PS and swine sources, such as serotypes *choleraesuis*, *typhimurium* and *heidelberg* though the genus *Salmonella* comprises more than 2,400 serotypes [27]. Due to its rapidity and sensitivity, however, this PCR can be useful in a *Salmonella* spp. reduction program of swine production.

Previous study reported a PCR assay for *B. hyodysenteriae* on the basis of sequence analysis of a recombinant clone designated pRED3C6, with a sensitivity between 1 and 10 organisms per 0.1 g of feces [6]. Sensitivity of the PCR in this study was 50 pg of DNA: a little lower than that of Elder

et al. [6]. Further studies are required to increase the sensitivity, and examine the specificity with others such as *B. pilosicoli*, *B. intermedia* and *B. murdochii*.

Elder *et al.* [7] also developed a multiplex PCR for detection of *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. based on the results of a single PCR of each organism [6,17,26]. The sensitivity and specificity of multiplex PCR results compared with the results of standard culture/histopathology for detection of the 3 bacterial agents from a total of 79 porcine intestinal specimens were 100%. The detection limit of its single PCR was higher than that in this study. However, that of the multiplex PCR could not be compared to each other because it was not tested in the earlier report. The present study developed a multiplex PCR assay to detect *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. simultaneously from porcine intestinal specimens based on the results of a single PCR of each organism. The major advantages of multiplex PCR over conventional PCR are the conservation of reagents (such as polymerase) and template, and the reduction in preparation and analysis time required to identify multiple target sites in 1 assay as opposed to running separate analysis for each target. Attempts to detect each species by multiplex PCR had some difficulties in setting the PCR condition such as annealing temperature and the numbers of PCR cycles. We first set 52°C as the annealing temperature when designing each single PCR primer set, and tested the broad range between 50–60°C in each PCR. The results showed best condition between 54–56°C of annealing temperature, but unclear band patterns with 50°C and 60°C depending on the single PCR (data not shown). The most specific and clear band was detected at 56°C when the multiplex PCR was tested between 52–58°C. Results of a single PCR assay could detect as little as 100 pg, 50 pg and 10 pg of purified chromosomal DNA from *L. intracellularis*, *B. hyodysenteriae* and *S. typhimurium*, respectively. However, the multiplex PCR resulted in a sensitivity of 10 times lower with *S. typhimurium* and same range with *L. intracellularis* and *B. hyodysenteriae*. This result might be explained by resulting from the combination of 3 primer sequences with a template from certain other organisms or by interference from PCR inhibitors.

There has been little recent information on the prevalence of 3 major enteric organisms affecting finishing pigs in Korea, specially no data for *B. hyodysenteriae*. We have developed this multiplex PCR to determine the prevalence of those organisms mentioned above with clinical field samples, which will be published later. The present study applied this method to clinical samples suspected of having PPE, SD and PS alone or in combination whether the multiplex PCR was available for screening the prevalence of *L. intracellularis*, *B. hyodysenteriae* and *Salmonella* spp. on pig farms. The accuracy for the detection of each organism using multiplex PCR results was increased compared with

the results of standard culture of *Salmonella* spp., *B. hyodysenteriae* and of histopathological examination of tissues for PPE. One specimen that was negative with *L. intracellularis* in feces but detected in mucosal specimen by PCR analysis might be explained by the previous reports of sensitivity differences between sources or effect of storage of feces at -80°C on subsequent DNA extraction and PCR amplification [19]. It had been reported that the PCR assay could detect $10^3\text{--}10^4$ *L. intracellularis* organisms/g of feces and 10^1 organisms/mucus [7].

In conclusion, the use of the multiplex PCR techniques for simultaneous detection and identification of 3 major enteric bacterial pathogens could be applicable to screening for 1 or more of these bacteria in intestinal specimens obtained from individuals or groups of pigs. Moreover, development of this multiplex PCR assay might provide results equivalent or superior to those of standard diagnostic methods, and be a useful alternative to single assays. Also, this would reduce the time, labor and expenses associated with nonspecific culturing of large numbers of intestinal specimens submitted for diagnostic investigation.

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