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Rapid detection of *Brachyspira hyodysenteriae* in swine intestinal specimens by PCR

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Abstract

Swine dysentery caused by *Brachyspira hyodysenteriae*, an anaerobic, beta-hemolytic spirochete, is a severe mucohemorrhagic diarrheal disease that primarily affects pigs during the growing and finishing period. The current standard laboratory procedure to culture and identify *B hyodysenteriae* takes 3 to 7 days. This report present a rapid PCR for detection *B hyodysenteriae* in a single reaction using DNA from swine intestinal samples. The PCR produced a specific 421bp PCR product with template DNA purified from *B hyodysenteriae,* and the accuracy for detection of *B hyodysenteriae* by PCR results compared with those of conventional method was 100% in intestinal specimens. Nonspecific bands were not detected with *B innocens*, a nonpathogenic common inhabitant spirochete, including other enteric bacterial organisms. This procedure could detect as little as 50 pg of template DNA for *B hyodysenteriae.*

Key words : *Brachyspira hyodysenteriae*, Swine dysentery, Diagnosis, PCR

Introduction

Swine dysentery(SD) caused by *Serpulina hyodysenteriae*, an anaerobic, beta-hemolytic spirochete, is a severe mucohemorrhagic diarrheal disease that primarily affects pigs during the growing and finishing period. The strongly beta-hemolytic *Treponema hyodysenteriae* was first identified and named by Harris *et al*[1], and the weakly beta-hemolytic *T innocens* by Kinyon and Harris[2], which was recently included in the genus *Brachyspira*[3].

Clinical signs of SD seem to occur in a cyclic manner. Diarrhea is the most consistent sign of SD. As the diarrhea progresses, watery stools containing blood,

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mucus and shreds of white mucofibrinous exudate are seen, with concurrent staining of rear quarters. Although the mechanism of tissue destruction has not been clearly elucidated, two toxins of *B hyodysenteriae*, lipopolysaccharide protein and hemolysin, have been described and characterized that may play a role in lesion development. In large groups of pigs affected with the disease, symptoms may reappear at 3 to 4 week intervals. This reappearance of symptoms often occurs only after removal of therapeutic levels of drugs from water or feed[4].

SD causes a tremendous financial loss due to death of pigs, decreased rate of growth, poor feed conversion and expenses for chemotherapy. For example, 36% of Austrian sow herds were said to be infected at a cost of about $100 per sow annum[5]. Similar findings(40%) have been recorded in the mid west of the USA[6], whereas in the UK a postal survey of veterinary practices indicated that 18.3% of breeding herds had the disease[7].

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 diarrhea or no diarrhea and in which the numbers of *B hyodysenteriae* isolates in the feces are far fewer than in swine with acute SD 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 difficult.

As mentioned above, SD is acknowledged as important cause of suboptimal performance and mortality in grower-finisher pigs and lots of studies on diagnostic methods of the disease have been known overseas. However, there is relatively little published information on PCR for detection of *B hyodysenteriae* in Korea. The purpose of this study is to develop a PCR for detection of the etiologic agents associated with SD in a single reaction using DNA from swine intestinal samples.

Materials and Methods

Bacterial strains(DNA)

A total of 10 *Brachyspira* spp including *B hyodysenteriae* B204(ATCC 31287), *B innocens* (ATCC 29796) and 8 field isolates of *B hyodysenteriae*, and 3 other enteric bacteria including *Escherichia coli* ML1410, *Campylobacter jejuni* and *Listeria monocytogens* were obtained from the National Veterinary Research and Quarantine Services(NVRQS), Anyang, Korea. All bacterial strains used in this study were identified biochemically and serologically[8].

Intestinal specimens

A total of 4 porcine intestinal specimens consisting of feces and mucosal scrapings were obtained from slaughtered pigs and field cases in 1999. Identification of the intestinal specimens for *B hyodysenteriae* was done by standard techniques[9].

Preparation of template DNA

Chromosomal DNA of *Brachyspira* spp and other bacterial strains used were purified as

previously described[10]. DNA from mucosal scrapings of swine intestinal specimens diagnosed as SD was extracted by the method described by Jones *et al*[11] and Boom *et al*[12]. The ileal mucosa from pigs with SD was scraped from the ileum and homogenized in tissue grinder. The homogenate was centrifuged at 750 g for 10 min at room temperature, and the supernatant was filtered sequentially through 5㎛, 1.2㎛ and 0.8㎛ filters. The filtrate was centrifuged at 8,000g for 10 min, and the pellet resuspended in PBS was referred to as the infected mucosal filtrate. A 20% diatomaceous earth suspension(50㎕) in 0.17 M HCl was vortexed with infected mucosal filtrate(50㎕) in a sterile microcentrifuge tube containing 950 ㎕ 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 held at room temperature for 10 min, vortexed, and centrifuged at 14,000 g for 20 sec. The lysis buffer was drawn off with a pipette, dried at 56℃ for 15 min and dissolved in TE buffer. After centrifugation at 12,000 g for 2 min, the supernatant was stored at -20℃. Fecal specimen(0.2g) 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㎕ of DE suspension. Further processing needed was the same as described above for the extraction of DNA from mucosal filtrate.

Oligonucleotides and PCR

The two primers for specific amplification of *B hyodysenteriae* defining a 421-bp DNA fragment by PCR assay were as follows : forward primer 5′-GCTGGAGATGATGCTTCTGG-3′ ; reverse primer 5′-GTCCAAGAGCTTGGCTGTTC-3′. *B hyodysenteriae* B204 *flaB*1 gene encoding a 38-KD flagella protein was selected for design of *B hyodysenteriae* specific primers[13]. The primer sets were designed using Primer 3 program(http:// www. genome.wi.mit.edu) and synthesized from Bioneer Co(Cheongju, Korea).

The 50 ㎕ of PCR mixture contained 5㎕ of 10×PCR buffer, 3㎕ of 25 mM $MgCl_2$, 4㎕ of 10 mM deoxynucleotide triphosphate mixture, 20 pmol of each primers, 1 ㎕ of each DNA template and 0.5 unit of *Taq* DNA polymerase(Takara, Co, Japan). PCR amplification was conducted on a DNA thermocycler (Robocycler, Stratagene, USA). The initial mixture was heated to 94℃ for 5 min. This step was followed by 45 cycles, each consisting of denaturation at 95℃ for 30 sec, annealing at 56℃ for 30 sec and polymerization at 72℃ for 1 min, followed by additional polymerization at 72℃ for 5 min. The presence of PCR products was determined by electrophoresis of 5㎕ 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.

Cloning and sequencing of PCR product

To confirm the identity of the PCR products, those were purified by using GeneClean II kit(Invitrogen, Carlsland, USA) after agarose gel electrophoresis and then cloned into *p*Bluescript KS plasmid in retriction enzyme *Eco* RV site. The 8 cloned each PCR products were sequenced by PCR sequencing method with Top™ DNA sequencing kit(Injae Co. Cheongju, Korea).

The identities of the products were con-

firmed by comparison of the sequence with previous report obtained from the GenBank[13].

Results

Specificity and sensitivity of PCR

In order to analyze the specificity of the PCR, DNA isolated from *Brachyspira* sp as well as from several other bacterial strains, including *E coli, C jejuni* and *L monocytogens* which cause intestinal diseases in swine, were used as a template DNA in the reactions. The PCR produced the expected 421bp DNA fragment with template DNA purified from *B hyodysenteriae,* and did not produce any nonspecific amplified DNA fragments derived from other intestinal organisms(Fig 1). To determine the minimal detectable concentration of *B hyodysenteriae* genomic DNA, PCR was conducted on serial dilutions of the template DNA from 10 ng to 10 pg. To increase the sensitivity of the PCR, template DNA diluted by 10 fold serial dilution was amplified with 40, 45, 50 and 55 cycles of PCR. The best amplification condition of the PCR was 45 cycles in PCR. There was no increase in sensitivity though 50 and 55 cycles of amplification showed the specific DNA fragments in the agarose gel. The assay could detect as little as 50 pg of template DNA for *B hyodysenteriae*(Fig 2).

Evaluation of clinical samples

A total of 4 swine intestinal samples diagnosed as SD by standard cultivation method were screened to determine the accuracy of the PCR for detection of *B hyodysenteriae* infection(Table 1). All specimens had PCR results that corresponded to the results of standard culture method in feces and mucosal scrapings. The sensitivity and specificity of the

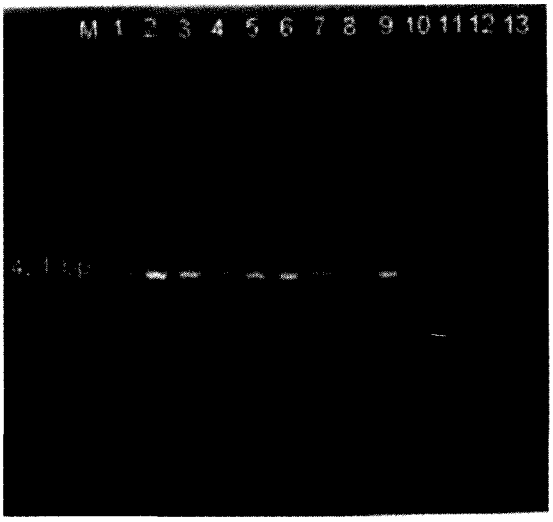

Fig 1. PCR amplification with choromosomal DNA for detection of *B hyodysenteriae*
M : *ϕX174* digested by *Hae III* ; Lane 1 : *B hyodysenteriae* B204 ; Lane 2-9 : *B hyodysenteriae* isolates ; Lane 10 : *S innocens* ; Lane 11 : *Campylobacter jejuni* ; Lane 12 : *Escherichia coli* ; Lane 13 : *Listeria monocytogens*.

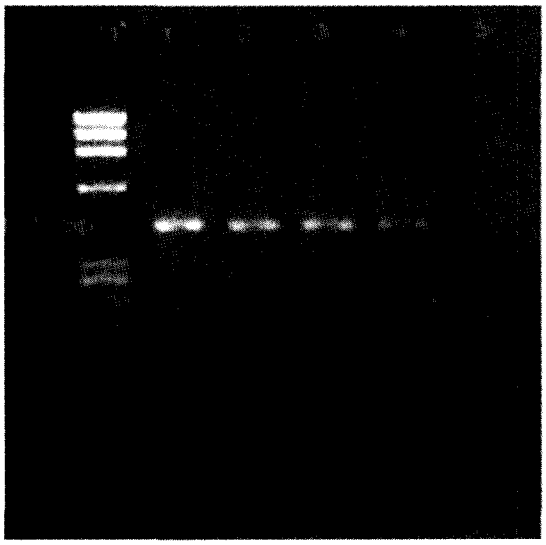

Fig 2. Sensitivity of PCR amplified DNA from *B hyodesenteriae*. Each template DNA was serially diluted.
M : *ϕX174* digested by *Hae III* ; Lane 1 : 10 ng of template DNA ; Lane 2 : 1 ng of DNA ; Lane 3 : 100 pg of DNA ; Lane 4 : 50 pg of DNA ; Lane 5 : 10 pg of DNA.

Table 1. Comparison of standard culture method with PCR for detection of *B hyodysenteriae* in specimens*

No of specimens	Results	
	Standard culture method	PCR
1	+	+
2	+	+
3	+	+
4	+	+
Total	4	4

*Specimens include feces and mucosal scrapings.

PCR results compared with the results of standard culture of mucosal scrapings and feces for *B hyodysenteriae* were 100%.

Discussion

The advent of molecular techniques has allowed for development of more rapid diagnostic test of pathogenic organism. Previous detection of *B hyodysenteriae* has been laborious and lacking in sensitivity and specificity. Also, the sensitivity of direct culture methods depends on the numbers of organisms present in the sample, which in turn depends on the stage of infection at the time of collection. Use of PCR has been accepted for the definitive identification of organism with high specificity and sensitivity. Recently, PCR method for detection of several pathogenic organisms has been reported[14~16]. Recombinant DNA technology has been used to generate oligodeoxynucleotide probes specific to the 16S rRNA of *B hyodysenteriae*, but the sensitivity of the probe method in feces was equivalent to that of routine bacteriological culture: 10^6 organisms per gram of feces[17]. Elder *et al*[16] reported a PCR for *B hyodysenteriae* on the basis of sequence analysis of a recombinant clone designed pRED3C6, with a sensitivity between 1 and 10 organisms per 0.1 g of feces. Jung *et al*[18] also used a hemolysin gene to select a probe specific for *B hyodysenteriae*. Its detection limit was estimated to be 100 pg of chromosomal DNA of *B hyodysenteriae*.

In this report, *B hyodysenteriae* strain B204 and all field isolates of *B hyodysenteriae* produced positive signs as determined by gel electrophoresis and sequencing of the PCR products. Nonspecific PCR products were not detected with *B innocens*, a nonpathogenic common inhabitant spirochete, including other untargeted enteric bacterial organisms. Results from this study indicated that The PCR could be used in diagnostic method for identifying *B hyodysenteriae*.

Sensitivity of the PCR in this study was 50 pg of DNA: a little higher compared with the detection limit of 100 pg by Jung *et al*[18], but lower than that of Elder *et al*[16]. Further studies are required to increase the sensitivity, and examine the specificity with others such as *B pilosicoli, B intermedia* and *B murdochii*.

SD has been widely distributed throughout the world, about 18~36% of pig herds being infected overseas[5~7]. Since first outbreak report of SD at Kimhae in 1975 by Bak *et al*[19], It has been known that about 17~30% of pig herds in Korea were infected with *B hyodysenteriae*[18,20]. SD causes a tremendous financial loss due to death of pigs, decreased rate of growth, poor feed conversion and expenses for chemotherapy. For example, 36% of Austrian sow herds were said to be infected at a cost of about $100 per sow annum[5]. We applied this method to clinical samples diagnosed as SD by culture technique whether this PCR could help

reduce transmission of *B hyodysenteriae* between farms. The accuracy for detection of *B hyodysenteriae* by PCR results compared with those of conventional method 100% in feces and intestinal mucosal scrapings, respectively.

So, this PCR technique might be useful as a method of identifying subclinically affected animals in herds and epidemiological studies of SD.

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