RESEARCH ARTICLE

Quantitative Detection of *Brachyspira hyodysenteriae* from Swine Dysentery Lesions by Using Direct Polymerase Chain Reaction (PCR) Method

Kyaw Kyaw Moe[1,2*], Michio Muguruma[3], Naoaki Misawa[2]

Abstract

Objective—We conducted experiments on the detection of *B. hyodysenteriae* from the clinical specimen of slaughtered pigs which were suspected of having SD infection by bacteriological diagnostic method and molecular biological method, and we investigated if the direct PCR assay using fecal samples is suitable for the rapid detection of *B. hyodysenteriae*.

Materials and Methods—A total of 52 intestinal mucosal and fecal specimens from pigs suspected of having SD in a local meat inspection centre in Japan were collected during April and May, 2005. Intestinal mucosa samples were used for the bacteriological isolation and identification. After identification, a number of organisms in one gram of intestinal mucosa or intestinal contents were determined. Genomic DNA extracted from 52 intestinal mucosal and fecal samples of slaughtered pigs were evaluated for molecular biological detection using *B. hyodysenteriae* specific NOX1 and F3B3 PCR primers.

Results—*B. hyodysenteriae* was isolated as 41/52 (80%) from clinical intestinal mucosal samples by conventional method while direct PCR method detected 35/52 (67%) from infected mucosa and fecal samples. The detection limit of bacteriological isolation method is higher than that of PCR method according to the results as mentioned above. Although the detection limit of the PCR method is quite different between primers and also clinical samples used, PCR technique could save the handling time needed and manipulate large amount of clinical samples at the same time.

Conclusion—Detection of *B. hyodysenteriae* isolated from pig fecal samples by using direct PCR method is the useful tool for the diagnosis of SD not only in pig farms and but also in meat inspection procedure.

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Keywords: Swine dysentery; *Brachyspira hyodysenteriae*; Molecular detection; Direct PCR method

[1]Department of Pathology and Microbiology, University of Veterinary Science, Yezin, Nay Pyi Taw, Myanmar

[2]Laboratory of Veterinary Public Health, Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki, Japan

[3]Laboratory of Food Science, Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, Japan

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***Corresponding author** E-mail: moethar@gmail.com

Introduction

Swine Dysentery (SD) was first described in 1921 in Indiana, USA by Whiting et al. [1]. The disease spread worldwide and remains important in pig-rearing countries because this disease affects pigs in the final stage of growth, causing persistent diarrhea and significant financial losses due to mortality, decreased rate of growth, poor feed efficiency and costs for antimicrobial therapy. The causal agent of SD is a spirochete which was first identified and named *Treponema hyodysenteriae* by Harris et al in 1972 [2]. The genus name was changed to *Serpulina* [3]. Later, due to phylogenetic rules, the genus name was again changed to *Brachyspira* [4] and the current designation of the etiological agent of SD is *Brachyspira hyodysenteriae*. *B. hyodysenteriae* is typically described as a gram-negative, anaerobic, loosely coiled, motile and strongly hemolytic spirochete. Six species of *Brachyspira* have been described taxonomically. *B. hyodysenteriae,* causal agent of swine dysentery; *B. intermedia*, causal agents of typhlitis/colitis in both poultry and pigs; *B. alvinipulli*, a cause of diarrhea in chicken; and *B. pilosicoli*, the agent of intestinal spirochetosis in pigs and also infects a number of other species including human, poultry and dogs; are considered pathogenic. *B. innocens* and *B. murdochii* are considered harmless commensals [5].

SD, a severe mucohaemorragic diarrheal disease is characterized by extensive inflammation and necrosis of epithelial surface of the large intestine in pigs [6]. The first evidence of the disease is usually soft, yellow to grey feces, but the consistent sign of SD is bloodstained, mucoid diarrhea. Appearance of white mucofibrinous grains in the stool is almost pathognomonic as the disease progresses.

The disease is usually introduced into a herd by carrier pigs that have recovered from a previous infection [7]. Such pigs may continue to shed the infectious agent for months, without manifesting clinical signs [8,9]. Moreover, wild rodents may be carriers of *B. hyodysenteriae* [10,11].

Strong hemolytic activity on blood agar plates has traditionally been the most important feature that has been used to identify pathogenic *Brachyspira* strains in routine diagnostics. The importance of the hemolysin as a virulence factor has been emphasized by experimental infections in mice and pigs performed [12]. Several genes of the organism have been characterized and some of them localized on the physical map of the genome, e.g. genes encoding for 16S rRNA [13], periplasmatic flagella subunits *flaA, flaB* [14-16] , hemolysin [17], and NADH oxidase gene (nox) [18].

As *B. hyodysenteriae* and other *Brachyspira* species need fastidious anaerobic growth requirements, have slow-growing nature and have closed phenotypic similarities amongst them, the development of rapid species-specific diagnostic protocols has been carried out in medical and veterinary diagnostic laboratories. Polymerase chain reaction (PCR) methods have been developed for detection of pathogenic species of *Brachyspira* [5,19,20]. Difficulties have been encountered when trying to extract spirochetal DNA directly from feces for use as a PCR template for direct detection of *B. hyodysenteriae* from feces [21]. In Japan, SD caused by *B. hyodysenteriae* is a major concern and its control is monitored by the Domestic Animal Infectious Diseases Control Law [22]. Carcasses which show lesions typical of SD have to be totally condemned as some regulations for meat inspection have been revised since February 2004. Diagnosis, prevention and treatment of swine dysentery and the routine works of meat

inspection of slaughtered pigs have recently become more important. To diagnose the swine dysentery infection in the slaughtered pigs, meat inspection officers inspect routinely by visual, bacteriological and histopathological examinations. Practically, bacteriological tests are laborious and time consuming. Therefore, there is the need to establish a rapid and reliable detection method of *B. hyodysenteriae* for the prevention of swine dysentery outbreak in pig farms and inspection of slaughtered pigs.

In this study, we conducted experiments on the detection of *B. hyodysenteriae* from the clinical specimen of slaughtered pigs which were suspected of having SD infection. The objectives of this study were to compare the detection limits of bacteriogical diagnostic methods and molecular biological methods and to investigate if the direct PCR assay using fecal samples is suitable for the rapid detection of *B. hyodysenteriae*.

Materials and Methods

Postmortem examination and sampling

Slaughtered pigs were observed at the postmortem examination for the presence of inflammation and hemorrhage in the large intestine, at a local meat inspection center in Miyazaki Prefecture, Japan. A total of 52 intestinal mucosa and fecal specimens were collected from pigs suspected of having swine dysentery infection. Another 50 intestinal mucosal and fecal specimens were also collected from those with no clinical abnormality. Large intestines (10cm long) were incised and examined again by the naked eye to confirm if mucohemorrhage was present or not. The intestinal mucosal scrapings and fecal contents were collected for the bacteriological and molecular biological tests. If necessary, mucosa specimens were examined by direct microscopy to confirm whether large spirochetes were present or not.

Bacteriological examination

One gram of each intestinal mucosa sample and 9ml of 10 mM phosphate buffered saline (PBS), pH7.2 were mixed by stomacher for 60 seconds. The mixture was serially diluted with PBS to obtain 10 fold dilutions. Serially diluted suspensions of each sample were inoculated onto BJ agar plates which is a selective media for *Brachyspira* species, consisting of Trypticase Soy Agar (40g/l), freshly thawed pig fecal extract (5%), Colistin (6.25μg/ml), Vancomycin (6.25μg/ml), Spectinomycin (200.0 μg/ml), Spiramycin (25.0 μg/ml), Rifampin (12.5 μg/ml), Sheep defibrinated blood (5%) (Kunkle & Kinyon, 1988) (23), and incubated anaerobically at 37˚C for 4 days with Anaeropack system (Mistubishi Gas Corporation, Tokyo, Japan).

After incubation, one colony showing film-like growth and hemolytic activity was selected randomly from the plates with the highest dilution and subcultured onto new BJ plates. The isolates were subjected to biochemical tests for the identification of *B. hyodysenteriae*. The isolates showing ambiguous biochemical characteristics, were confirmed by PCR techniques for the specific detection of *B. hyodysenteriae, B. pilosicoli*, and *B. innocens* as described elsewhere [Ayteo et al., 1999, Elder et al., 1997, La et al., 2003] (5, 20, 24). After identification of the isolates as *B. hyodysenteriae*, the

number of organisms in one gram of intestinal mucosa or intestinal contents was determined. The isolated *Brachyspira* was stored in 1ml tube containing Mist Desiccans medium (400 ml of this medium contains 3gm of Trypticase Soy broth, 30gm of glucose, 100ml of D/W and 300ml of horse blood) at -80˚C until further tests.

Identification of isolates

Brchyspira isolates were identified by performing the following tests:

Beta hemolysis. One pure colony from the plate of the best dilution ratio was inoculated onto Trypticase soy agar plate containing 5% defibrinated sheep blood and incubated anaerobically at 37˚C for 4 days. After incubation, the blood agar plate was examined and beta hemolysis was classified as weak or strong.

Gram staining. Primary growth of *Brachyspira*-like colonies on the blood agar plates were Gram stained and examined for motility by phase-contrast microscopy. Gram-negative, loosely coiled motile organisms were subjected to further biochemical tests.

Indole production. 50µl of indole reagent (0.5%L-tryptophan, 0.2%K_2HPO_4,pH 7.8) and 50µl of bacterial suspension (PBS containing spirochetal cells freshly harvested from a blood agar plate) were added to the well of a microtitre plate and incubated aerobically at 37˚C for 4 hours. Then 25µl of Kovac's reagent was added and color changes were checked after 10 min.

Hippurate hydrolysis. 50µl of 1% sodium hippurate solution was mixed with 50µl of spirochetal cells suspension and incubated aerobically at 37˚C for 4 hours. Then 25µl of ninhydrine solution (ninhydrine in equal parts of acetone and butanol) was added to the above mixture and any change in color was recorded after 30 min. The reaction was judged to be positive if a deep blue or purple color developed and negative if the solution turned light blue or remained colorless.

Bacterial enzyme activity (α-glucosidase, β-glucosidase, α- glactosidase). 50µl of the substrates listed below dissolved in 5% PBS solution were added to the microtitre plates respectively. Then 50µl of fresh bacterial cells suspension was added and incubated at 37˚C for 4 hour and any changes in color was recorded.

p-nitrophenyl-α D-glucopyranoside for α-glucosidase actvity
p-nitrophenyl-β D-glucopyranoside for β-glucosidase activity
p-nitrophenyl-α D-galactopyranoside for α-galactosidase activity

Confirmation of bacterial isolates by PCR amplification

To confirm the result of identified bacterial isolates of intestinal mucosal samples, DNA was extracted from the isolated pure cultures by the phenol/chloroform method (25) and PCR tests were performed as follows;

DNA extraction. Bacterial cells were harvested from a pure culture from BJ agar plate and resuspended in a microcentrifuge tube containing 567µl TE buffer by repeated pipetting. 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K were added to give a final concentration of 100µl/ml proteinase

K in 0.5% SDS, mixed thoroughly and incubated at 37˚C for 1 hr. 100µl of 5M NaCl was added and mixed thoroughly. Then 80µl of CTAB/NaCl solution was added, mixed thoroughly and incubated at 65˚C for 10 min. An approximately equal volume (0.7 to 0.8 ml) of chloroform isoamyl alcohol (24:1) was added, mixed thoroughly and spinned at 13000 rpm for 5 min. The aqueous, viscous supernatant was transferred to a new microcentrifuge tube and an equal volume (0.7ml) of phenol/choloroform/isoamyl alcohol (25:24:1) was added and spinned at 13000 rpm for 5 min. The supernatant was transferred to a new tube and 0.6 volume (240µl) of isopropanol was added to precipitate the nucleic acids. The tube was shaken back and forth until a stringy white DNA precipitate was clearly visible. At this point, the supernatant was discarded by using a paper towel and the pellet was washed with 1ml of 70% ethanol at 13000 rpm for 1min. The supernatant was carefully removed by using a paper towel and the tube was spinned at 13000 rpm for 5 min with cover open to dry. Finally, 100µl of TE buffer was added to elute DNA. The concentration of DNA was measured with ND 1000 Spectrophotometer (NanoDrop, DE, USA) and stored at -20˚C before use.

PCR Amplification with B. hyodysenteriae specific NADH oxidase gene (nox) Nox 1 primers. PCR was performed with NOX1 primers (5) for the identification of *B. hyodysenteriae* isolates from intestinal mucosal samples of infected and normal pigs. Genomic DNA extracted from bacterial isolates of intestinal mucosal samples of infected pigs were used as templates for PCR test. The PCR was performed in a 20µl reaction mixture containing 20ng of genomic DNA, 2µl of x 10 PCR buffer (Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer (Forward Primer SHF and Reverse Primer SHR). The reaction was initiated by denaturation at 95˚C for 1 min and cycled 30 times at 95˚C 30s, annealed at 45˚C for 1min, 72˚C for 2min with a final annealing at 48˚C and final extension at 72˚C for 10min. PCR products were examined by Agarose gel electrophoresis for 20 min in 1% (wt/vol) agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light.

Brachyspira innocens specific NADH oxidase gene (nox) NOX 4 primers. PCR was performed with NOX4 primers (5) for the identification of *B.innocens* isolates from intestinal mucosal samples of normal pigs. Genomic DNA extracted from bacterial isolates of intestinal mucosal samples of normal pigs were used as templates for PCR test. The PCR was performed in a 20µl reaction mixture containing 20ng of genomic DNA, 2µl of x 10 PCR buffer (Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer (Forward Primer SINNF and Reverse Primer SINNR). The reaction was initiated at 95˚C for 1 min and cycled 30 times at 95˚C for 30s, annealed at 46˚C for 1min, 72˚C for 2min with a final annealing at 46˚C and final extension at 72˚C for 10min. PCR products were examined by Agarose gel electrophoresis for 20 min in 1% (wt/vol) agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light.

16S rRNA sequencing

Two bacterial isolates from intestinal mucosa of pigs suspected of having swine dysentery showed *Brachyspira-like* colonies structure and biochemical activities but the DNA templates of which could not be amplified by *B. hyodysenteriae* specific NOX1 primers. Therefore, these two isolates were identified by direct sequencing with 16S ribosomal RNA primers.

Amplification of 16S rRNA. Genomic DNA of the above two bacterial isolates were used as templates and PCR of the 16S rRNA gene was performed with the primers 8F (5'-AGA GTT TGA TCM TGG CTC AG 3') and 15R (5'-AAG GAG GTG ATC CAR CCG Ca-3'), which were designed based on the *Escherichia coli* 16S rRNA numbering system (26). The PCR was performed in a reaction mixture of 25µl containing 20ng of genomic DNA, 2.5µl of 10x PCR buffer(Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo, Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer. The reaction was initiated at 95˚C for 10 min and cycled 35 times at 95˚C 30s, annealed at 55˚C for 30s and 72˚C for 30s with final extension at 72˚C for 10min.

16S rRNA sequence data analysis. The fragment for sequencing was amplified by PCR and the products were purified by using QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The nucleotide sequence was determined directly from PCR fragment in a PCR-based reaction using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems Division) and analyzed using the PE Applied Biosystems 310 DNA sequencer (PE Applied Biosystems Division) at the University of Miyazaki Gene Research Center. Computer analyses of the DNA sequences were performed with the Genetics Computer Group programs; database similarity searches were performed through the National Center for biotechnology Information using the BLASTX algorithm.

DNA extraction from intestinal mucosa and fecal samples

Genomic DNA was extracted from 52 intestinal mucosa and fecal samples of slaughtered pigs with SD and 50 intestinal mucosa and fecal samples of slaughtered pigs with no clinical abnormality by using QIAamp DNA stool minikit (Qiagen K.K, Japan) according to the manufacturer's instructions. An amount of 180-220 mg of intestinal mucosa or feces was added to a 2 ml microcentrifuge tube and resuspended in 1.4 ml of Buffer ASL by vortexing continuously for 1 min or until the sample was thoroughly homogenized. The suspension was incubated for 5min at 70˚C, vortexed for 15 sec and centrifuged at 20,000xg for 1 min to pellet particles. The supernatant (1.2 ml) was transferred into a new 2 ml microcentrifuge tube and the pellet was discarded. An InhibitEX tablet was added to each sample and vortexed immediately and continuously for 1 min or until the tablet was completely suspended. This suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. The tube was centrifuged at 20,000X, g for 3 min to pellet inhibitors bound to InhibitEX. All the supernatant was placed into a new 1.5 ml tube and centrifuged at 20,000xg for 3 min. The supernatant (200µl) was added to a new 1.5 ml centrifuge tube containing 15µl of Proteinase K. Then 200µl of Buffer AL was added and vortexed for 15 sec before incubation at 70˚C for 10 min. 200µl of ethanol (96-100%)

was added to the lysate and mixed by vortexing. The complete lysate was carefully applied to a QIAamp spin column without moistening the rim and centrifuged at 20,000x g for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube and 500µl of Buffer AW1 was added to the column and centrifuged at 20,000x g for 1min. Then the QIAamp spin column was placed in a new 2 ml collection tube and washed with 500µl of Buffer AW2 at 20,000X g for 3 min. The QIAamp spin column was transferred into a new 1.5 ml microcentrifuge tube. 200µl of Buffer AE was added and incubated for 1 min at room temperature. The tube was centrifuged at 20,000xg for 1 min to elute DNA.

The concentration of each DNA template extracted from the intestinal mucosal samples and fecal samples was measured with ND 1000 Spectrophotometer (NanoDrop, DE, USA) and stored at -20˚C until use.

PCR screening tests of intestinal mucosa samples and fecal samples of 52 infected slaughtered pigs

The names of primer pairs used in this study are shown in **Table 1**.

Table 1. PCR Primers Used in This Study

No.	Name of primer	Agent Name	Oligonucleotide sequence (5'-3')	Tm (˚C)	Product Size (bp)	Reference
1.	NOX1 SHF	*B. hyodysenteriae*	TTA AAA CAA GAA GGA ACT ACT	40	821	5
	SHR		CTA ATA AAC GTC TGC TGC	50		
2.	F3Hyo	*B. hyodysenteriae*	CTG GGC TAA CAA AAA ATT ATG	47	198	This study
	B3Hyo		ATA ATT TCT TGT CCT TGC TGAT	47		
4.	NOX4 SINNF	*B. innocens*	CCT GAA AGT TTA AAA GCTG	52	729	5
	SINNR		CAG TGT ATT CTT CTT TTCC	52		
5.	P1	*B. pilosicoli*	AGA GGA AAG TTT TTT CGC TTC	49	**823**	**20**
	P2		GCA CCT ATG TTA AAC GTC CTTG	51		

B. hyodysenteriae specific NADH oxidase gene (nox) Nox 1 primers. Genomic DNA extracted from 52 intestinal mucosa samples and 52 fecal samples of infected pigs were used as templates for direct PCR screening in these assays. The PCR was performed in a 20µl reaction mixture containing 20ng of genomic DNA, 2µl of 10x PCR buffer (Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer (Forward Primer SHF and Reverse Primer SHR). The reaction was initiated by denaturation at 95˚C for 1 min and cycled 30 times at 95˚C for 30s, annealed at 45˚C for 1min, 72˚C for 2min with a final annealing at 48˚C and final extension at 72˚C for 10min. PCR products were examined by Agarose gel electrophoresis for 20 min in 1% (wt/vol) agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light.

B. hyodysenteriae specific F3B3 primers. Genomic DNA extracted from 52intestinal mucosa samples and 52 fecal samples of infected pigs were used as templates for direct PCR screening in these

assays. The PCR was performed in a 20µl reaction mixture containing 20ng of genomic DNA, 2µl of 10x PCR buffer (Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer (Forward Primer F3 and Reverse Primer B3). The reaction was initiated by denaturation at 95˚C for 5 min and cycled 35 times at 95˚C for 30 sec, annealed at 42˚C for 30s, 72˚C for 1min with final extension at 72˚C for 10 min. PCR products were examined by Agarose gel electrophoresis for 20 min in 1% (wt/vol) agarose gels in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light.

Brachyspira innocens specific NADH oxidase gene (nox) Nox 4 primers. Genomic DNA extracted from intestinal mucosal samples were used as templates for direct PCR screening in this assay. The PCR was performed in a 20µl reaction mixture containing 20ng of genomic DNA, 2µl of 10x PCR buffer (Qiagen, Tokyo, Japan), 250µM of each Deoxynucleotides (Amersham Pharmacia Biotech, Tokyo Japan), 0.625 U of Taq DNA polymerase enzyme (Qiagen, Tokyo, Japan), and 160nM of each primer (Forward Primer SINNF and Reverse Primer SINNR). The reaction was initiated at 95˚C for 1 min and cycled 30 times at 95˚C 30 sec, annealed at 46˚C for 1min, 72˚C for 2 min with a final annealing at 46˚C and final extension at 72˚C for 10 min. PCR products were examined by Agarose gel electrophoresis for 20 min in 1% (wt/vol) agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light.

PCR screening tests of intestinal mucosa samples and fecal samples of 50 slaughtered pigs with no clinical symptoms

The PCR screening tests of intestinal mucosa samples and fecal samples of 50 healthy slaughtered pigs were also performed the same as SD suspected clinical samples mentioned above.

Examination of detection limit of PCR from DNA of intestinal mucosal samples spiked with B. hyodysenteriae

To assess the sensitivity of direct PCR assay for the intestinal mucosal extractions, the detection of *B. hyodysenteriae* from an artificially contaminated intestinal mucosal sample of pig was performed.

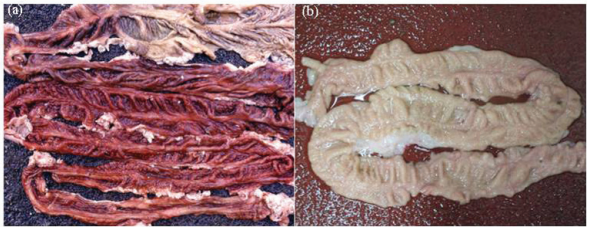
A fresh intestinal mucosal sample was diluted to 2% with 10 mM phosphate buffered saline (PBS) (pH 7.2). The bacterial cells from isolated pure culture were diluted serially and seeded into dilute intestinal mucosal samples at concentrations ranging from 10^1 to 10^6 cfu/ml. DNA was extracted from the spiked intestinal mucosal samples by using QIAamp DNA stool minikit (Qiagen K.K, Japan) and PCR tests were performed separately by using *B. hyodysenteriae* specific NOX1 and F3B3Hyo primers with the same PCR programs as described above.

Results

Necropsy findings

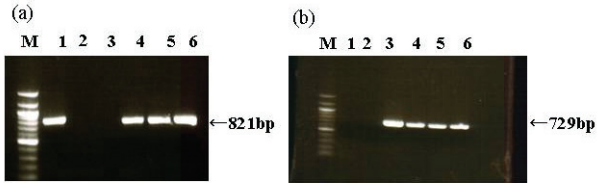
Among 102 slaughtered pigs, the large intestines of 52 pigs showed typical lesions of swine dysentery; extensive inflammation and hemorrhage (**Figure 1**). The mesentery and colonic tissues were edematous. Bloody mucous were also found in the large intestinal content of these pigs and large motile spirochetes were seen in direct microscopy. Gross lesion of mucosal surface of the colon varied from mucosal hyperemia and catarrhal inflammation to pseudo- membrane accumulation and necrosis. No pathological gross lesions were seen in another 50 large intestinal specimens of slaughtered pigs (**Figure 1**).

Figure 1. Lesions of Intestinal Specimens[a]



[a](a) Mucosal hemorrhage and inflammation of opened large intestine of infected pig (b) Normal intestinal specimen of healthy slaughtered pig

Figure 2. PCR Products Amplified by Specific Primers[a]



[a](a) PCR products amplified by *B. hyodysenteriae* specific NOX1 primers. M; 100 bp molecular size marker, Lanes: 1, positive control of *B. hyodysenteriae*; 2, *B. pilosicoli*; 3, *B. innocens*; 4-6, DNA from isolated culture samples (b) PCR products amplified by *B. innocens* specific NOX4 primers M; 100 bp molecular size marker, Lanes: 1, *B. hyodysenteriae*; 2, *B. pilosicoli*; 3, positive control of *B. innocens*; 4 to 6, DNA from isolated culture samples

Isolation of B. hyodysenteriae from intestinal mucosa samples of slaughtered pigs with clinical symptoms of swine dysentery

Brachyspira-like colonies were seen on BJ agar plates after 72 hours under anaerobic incubation at 37˚C. Forty three *Brachyspira*-like bacteria were isolated from intestinal mucosal samples out of 52 samples of slaughtered pigs with clinical symptoms of Swine dysentery. Strong hemolysis was seen on blood agar plates after 72 hours after anaerobic incubation at 37˚C. All of the bacterial cultures showed large Gram-negative spirochetes under contrast microscopy. Bacterial counts ranged from 10^2-10^7 cfu/g (**Table 2**). Biochemical test results showed that these spirochetes belonged to *B. hyodysenteriae* by indole production, hippurate hydrolysis, α glucosidase and β glucosidase activity, but lack of α galactosidase activity. However, the results of PCR amplification

Table 2. Quantitative Isolation of From *B. hyodysenteriae* Intestinal Mucosa of Pigs with Swine Dysentery Infection and *B. innocens* From Intestinal Mucosa of Normal Pigs

Bacterial count (cfu/g)	Number of *B. hyodysenteriae* isolates from infected mucosa	Number of *B. innocens* isolates from nomal mucosa
10^2	1	2
10^3	12	2
10^4	5	5
10^5	6	2
10^6	7	2
10^7	10	-
Total isolates	41/52 (79%)	13/50 (26%)

Table 3. Species Confirmation of Isolates by PCR Amplification of DNA Extracted from Pure Culture Isolated from Intestinal Mucosa Samples of Slaughtered Pigs

Source	Number of isolates	Name of primer used	Name of causal agents	PCR positive
Infected pigs	43*	NOX1	*B. hyodysenteriae*	41* (95%)
Normal pigs	13	NOX4	*B. innocens*	13 (100%)

*Two isolates were PCR negative and 16S rRNA sequencing results showed that these isolates were *B. innocens* and *Campylobacter jejuni*.

showed that only 41(79%) DNA samples extracted from bacterial isolates were *B. hyodysenteriae* by producing a specific band (872 bp) in Agarose gel electrophoresis (**Table 3 and Figure 2**). The other two isolates were *B. innocens* and *Campylobacter coli* according to the direct sequencing result.

Isolation of B. innocens from intestinal mucosa samples of slaughtered pigs with no abnormalities

Brachyspiras-like bacteria were also isolated from 13 (26%) intestinal mucosal samples out of 50 samples collected from 50 slaughtered pigs with no fecal abnormality and bacterial counts ranged from 10^2-10^7 cfu/g (**Table 2**). Blood agar plates showed weak hemolysis produced by spirochetes. Biochemical test results also confirmed that these 13 *Brachyspira* isolates belonged to nonpathogenic *B. innocens*. The result of PCR amplification using NOX1 primers showed that the isolates from the intestinal mucosal samples of normal pigs were not *B. hyodysenteriae*. Furthermore, PCR amplification using *B. innocens* specific NOX4 primers revealed that these isolates were nonpathogenic *B. innocens* (**Table 3, Figure 2**).

Table 4. PCR Screening Test Results of Mucosa Samples and Fecal Samples of 52 Infected Slaughtered Pigs

Name of Primer	Agent	PCR positive	
		mucosa	feces
NOX1	*B. hyodysenteriae*	35 (67%)	20 (38%)
F3B3 Hyo	*B. hyodysenteriae*	33 (63%)	35 (67%)
NOX4	*B. innocens*	0	not done

Table 5. Comparison of Detection Limit of *B. hyodysenteriae* Between Bacteriological Diagnostic Method and PCR Method Using Two Primer Pairs (in 52 Infected Pigs)

Bacterial isolation	PCR	NOX1		F3B3	
		Mucosa	Feces	Mucosa	Feces
+	+	35	20	33	35
+	-	6	21	8	6
-	+	0	0	0	0
-	-	11	11	11	11

++; bacterial isolation positive, PCR positive

+- ; bacterial isolation positive, PCR negative

-+ ; bacterial isolation negative, PCR positive

- - ; bacterial isolation negative, PCR positive

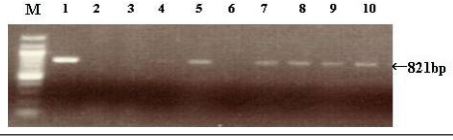
Dectection of B. hyodysenteriae from intestinal mucosa samples and fecal samples of 52 infected pigs

B.hyodysenteriae specific NADH oxidase gene (nox) NOX1 primers. The direct PCR assay with *B. hyodysenteriae* specific NADH oxidase gene (nox) NOX1 primers showed 35 positive results out 52 intestinal mucosa samples and 20 positive results out of 52 fecal samples (**Table 4 & 5 and Figure 3**)

B. hyodysenteriae specific F3B3 primers. The direct PCR assay with *B. hyodysenteriae* specific F3B3 primers showed 33 positive results out of 52 intestinal mucosal samples and 35 positive results out of 52 fecal samples (**Table 4 & 5 and Figure 4**).

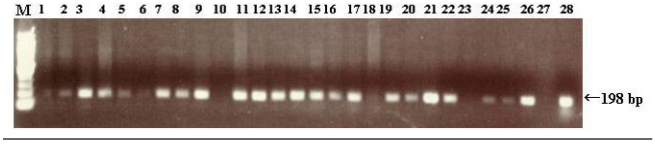
The Total number of pig from which we could detect *B. hyodysenteriae* by PCR was 38 (73%)

Figure 3. PCR Products of DNA from Infected Feces Amplified by *B.hyodysenteriae* Specific NOX1 Primer [a]



[a]PCR products of DNA from infected feces amplified by *B.hyodysenteriae* specific NOX1 primer. M- 100 bp DNA ladder; Lane 1, positive control; Lane 2 to 10, DNA from fecal samples of infected slaughter pigs

Figure 4. PCR Products of DNA from Infected Feces Amplified by *B.hyodysenteriae* Specific F3B3 Primer[a]



[a]PCR products of DNA from infected feces amplified by *B.hyodysenteriae* specific F3B3 primer. M- 100 bp DNA ladder; Lane 1 to 26, DNA from fecal samples of infected slaughter pigs; Lane 27, negative control; Lane 28, positive control

out of 52 slaughtered pigs using both mucosa and fecal samples with 3 different *B. hyodysenteriae* specific primer pairs, NOX1, F3B3 and H1H2.

Detection of Brachispira from intestinal mucosa samples of 50 normal pigs

B. hyodysenteriae specific NOX1 primers and *B. hyodysenteriae specific F3B3 primers.*Direct PCR assay by using DNA extracted from intestinal mucosa samples and fecal samples of 50 pigs without clinical signs and symptoms showed no positive results for these two *B. hyodysenteriae* specific primer pairs.

B. innocens specific NADH oxidase gene (nox) NOX 4 primers.

The direct PCR assay carried out with *B. innocens* specific NADH oxidase gene (nox) NOX 4 primers showed positive results in 2 intestinal mucosal samples out of 50 pigs without clinical signs and symptoms.

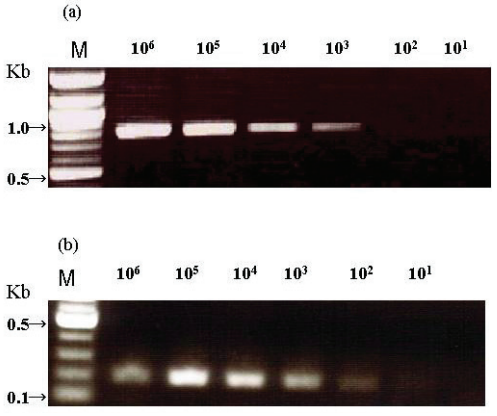
Detection limit of spiked intestinal mucosal samples

The sensitivity of the PCR assay was estimated on the basis of the number of spirochetal cells present in the original culture. The results of PCR using Nox1 primers showed that the minimum dilution concentration of spiked intestinal mucosa samples at which *B. hyodysenteriae* could be detected was 10^3cfu/ml while PCR with F3B3 primers showed that *B. hyodysenteriae* could be detected at the concentration of 10^2cfu/ml (**Figure 5**).

Discussion

Several selective media have been described for the isolation of intestinal spirochetes: S400 (spectinomycin) (27), CVS (colistin, vancomycin and spectinomycin) (28), BJ (spectinomycin, vancomycin, colistin, spiramycin and rifampin with swine fecal extract) (23) and BAM-SR (blood agar modified medium with spectinomycin and rifampin) (29). In a comparative study of these media, BJ proved

Figure 5. Comparison of PCR Specificity of Spiked Intestinal Mucosa Samples[a]



[a]Comparison of PCR specificity of spiked intestinal mucosa samples with (a) NOX1 primer, (b) F3B3 hyo primer. M; 100 bp molecular size marker, Lane 1to 6; intestinal mucosa samples spiked with 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 cfu/ml of *B. hyodysenteriae cells*.

to be the most efficient in eliminating normal fecal flora and enhancing growth of *B. hyodysenteriae* and *B. innocens* from fecal samples (30). The presence of spirochetes was determined by the examination of the surface growth of the bacteria on the plate that spread from the site of primary inoculation and formed a zone of strong β-hemolysis. In order to obtain a pure culture of *B. hyodysenteriae*, re-cultivaion on the same media was done (31).

We used BJ medium for isolation of *Brachyspiras* in this study. It took at least 8 days to isolate spirochetes on BJ medium, obtain pure cultures by cultivation on blood agar plates, and to test biochemical properties as these spirochetes have fastidious and slow-growing nature. Sometimes, it took a long time to obtain a pure culture because we needed to subculture at least for 3 times. It also took several hours to perform genomic DNA extraction from bacterial cells of pure cultures and PCR amplification for species confirmation.

Forty three *Brachyspira*-like bacteria were isolated although postmortem examination results showed 52 pigs had SD infection. On the other hand, the PCR amplification and 16S rRNA direct sequencing results revealed that only 41 (79%) of isolates were of *B. hyodysenteriae* species. From these results, we considered that we could not use the bacteriological tests as proper diagnostic method for the detection of the causal agent of SD.

Many researchers have tried to detect the *B. hyodysenteriae* from intestinal specimens, feces and mucosal scrapings by PCR technique including single PCR, Duplex-PCR and Multiplex-PCR [20,21,31]. La et al [20] carried out fecal extraction-duplex-PCR assay and proved that this method was specific when tested with a large number of intestinal spirochetes strains. Ateo et al [5] designed *B. hyodysenteriae* specific NADH oxidase NOX1 primers and *B. innocens* specific NADH oxidase NOX4 primers and detected *Brachyspiras* only from genomic bacterial DNA extracted from pure cultures. They suggested in their report that future work is required to investigate the feasibility of using these PCR tests for direct detection of the spirochetes in clinical samples [5].

In this study, we used *B. hyodysenteriae* specific NADH oxidase NOX1 primers and *B.*

innocens specific NADH oxidase NOX4 primers not only for the detection of *Brachyspiras* from genomic bacterial DNA extracted from pure culture but also for the direct detection of spirochetes in clinical samples of pigs.

We could detect 35 (67%) *B. hyodysenteriae* positive result out of 52 intestinal mucosal samples and 20 (38%) positive results out of 52 fecal samples of infected pigs by PCR amplification with NOX1 primers. We also detected 33(63%) positive result out of 52 intestinal mucosal samples and 35 (67%) positive results out 52 fecal samples of infected pigs by PCR with *B. hyodysenteriae* specific NADH oxidase F3B3 primer which was self-designed for this study. Direct PCR using F3B3 primers could detect *B. hyodysenteriae* from both samples with nearly the same detection limit of 33 (63%) positive results of mucosa and 35 (67%) positive results of feces. From these PCR results, F3B3Hyo primers showed the best sensitivity for direct PCR assay of *B. hyodysenteriae*. The comparison of intestinal mucosal spiking results also proved that the specificity of PCR assay with F3B3Hyo primers was higher than that of NOX1 primers (Fig. 5). From these facts, we considered it is better to use the primer pairs which can produce good detection limits in the direct detection of *B. hyodysenteriae* from clinical samples.

La et al [20] reported that difficulties have been encountered when trying to extract spirochetal DNA directly from feces for use as a PCR template. It is possible because the detection limit of the direct PCR detection of *B. hyodysenteriae* from infected fecal samples (35 positive results, 73%) was less than that of bacterial isolation method (41 isolates, 79%).

However, compared to the bacterial isolation method, the direct PCR method is more feasible because spiking results for both primer pairs pointed out that the direct PCR method is reliable for the detection of *B. hyodysenteriae* even under the condition of low bacterial population in fecal contents (**Figure 5**). We could investigate the spirochetes in clinical samples within one day including the handling time needed for DNA extraction from intestinal mucosal and fecal samples by QIAamp DNA stool minikit. Moreover, direct PCR method can help to save the cost of laboratory consumables. In conclusion, we consider that we could apply this rapid detection method of *B. hyodysenteriae* DNA by direct PCR for the diagnosis and prevention of swine dysentery outbreak on pig farms by using pig fecal sample only.

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