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Identification of *Salmonella* spp. from porcine salmonellosis by matrix-assisted laser desorption ionization-time of flight mass spectrometry

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Abstract

A total of 41 *Salmonella* (*S.*) strains were isolated from pigs suffered with severe watery diarrhea and were tried to identify by both matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and polymerase chain reaction (PCR) analysis. Fibrinous exudate and ulceration in the large intestine were prevalent in gross observation, and variable degrees of enteritis were observed in the histology of large intestines. Subsequent polymerase chain reaction (PCR) analyses demonstrated that 41 strains were identified as *S.* Typhimurium (39 strains), though 2 stains were failed to identify. Further identification was performed using both direct smear and protein extraction method by MALDI-TOF MS analyses. In terms of extraction methods, 100% (41/41) of isolates were identified to species level of *S.* spp. Whereas only 43.9% (18/41) were identified to species level using the direct method. These results thus suggest that rapid and accurate diagnosis of porcine salmonellosis can be guaranteed by MALDI-TOF MS combined with protein extraction method.

Key words : Porcine, Salmonellosis, Identification, PCR, MALDI-TOF MS

INTRODUCTION

Salmonella (*S.*) spp. are Gram-negative, motile, facultative anaerobic and flagellated rod-shape bacilli that cause gastroenteritis, septicemia and foodborne poisoning in human and animal (van Duijkeren et al, 2002). Although more than 2,400 serotypes are widely distributed with broad host ranges, notable serotypes of *salmonella* in pigs are *S.* Typhimurium, *S.* Choleraesuis, and *S.* Typhisuis (Griffith et al, 2006; Brown et al, 2007). The pathology of porcine salmonellosis can be varied according to the causative agents (Wilcock et al, 1976; Griffith et al, 2006; Brown et al, 2007). For instance, pigs infected with *S.* Choleraesuis are reluctant to move, and mortal with cyanosis of extremities and abdomens. However, enterocolitis is the major pathological observation in pigs infected with *S.* Typhimurium. Studies have indicated that immunologically naïve pigs in poor hygiene are largely responsible for the outbreak of porcine salmonellosis (Griffith et al, 2006).

Since *S.* spp. has great economic importance in global swine industry, rapid diagnosis is essential for public health, cost-effectiveness and corresponding therapy (Griffith et al, 2006; Abubakar et al, 2007). Many studies have shown that methods including biochemical tests, VITEK 2 system, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) are effective to diagnosis for porcine salmonellosis (Baggesen et al, 1996; Griffith et al, 2006). Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), has been employed for the identification of the pathogenic bacteria

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in both human and veterinary medicine (Mellmann et al, 2008; Seng et al, 2009; Emonet et al, 2010; van Veen et al, 2010; Saffert et al, 2011; Welker and Moore, 2011). This technique is based on the generation of the spectral profile of proteins and peptides mostly derived from bacterial ribosome. Since specific peak patterns can be acquired from various sample preparation methods such as whole cells, cell lysates, and bacterial extracts from different bacterial species, MALDI- TOF MS has been shown to be a useful and simple method for not only rapid identification of bacteria but also discrimination among different clusters of microorganism based on proteome type (Hsieh et al, 2008; Sparbier et al, 2012). In this study, *Salmonella* spp. strains were isolated from large intestinal contents diagnosed as porcine salmonellosis by pathologic examination. Further identification of *Salmonella* spp. by MALDI-TOF MS was performed to compare with the results of PCR. The results indicated that MALDI-TOF MS is effective tools for identification of *Salmonella* spp. that responsible for porcine salmonellosis.

MATERIALS AND METHODS

Pathology and Microbiology

From 2007 to 2011, a total of 41 strains of *S*. spp. were isolated from the cases of porcine salmonellosis submitted to Pathology Department of Veterinary Medicine, Jeju National University. All 41 pigs showed wasting and watery or soft stool diarrhea. For bacterial culture, aseptically collected feces from large intestine of 41 pigs were inoculated on Rambach agar plates (Merck, Germany) and aerobically incubated for 48 h at 37°C. Subsequent biochemical test and Gram staining was performed to clarify the isolated bacteria. After necropsy of pigs, all major parenchymal organs were fixed in 10% phosphate-buffered formalin, routinely processed, embedded in paraffin, and stained with hematoxlyin and eosin (H&E) for light microscopic examination. Notably, intensive observation was carried out for the slides from large intestines, the target organ of porcine salmonellosis.

PCR analysis

Bacterial colonies from Rambach agar were dissolved in DNase-free distilled water (Invitrogen, USA), and centrifuged at 14,000×g for 10 min to discard the supernatant. The pellets were then re-suspended in 300 μL of water to incubate for 10 min at 100°C and kept at −20°C. After defrosting at room temperature, samples were centrifuged at 14,000×g for 10 min and supernatants were used for the template DNA. The three PCR analysis was carried out to differentiate *S*. spp. using oligonucleotide primer sets in Table 1 (Widjojoatmodjo et al, 1991; Chiu et al, 2005; Kim et al, 2006). All analyses were performed using a Dice TP600 PCR Thermal Cycler (TaKaRa, Japan). PCR amplification for *S*. spp. was an initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 30 sec, annealing at 52°C for 30 sec, 72°C for 1 min, and finished with a final extension at 72°C for 5 min and stored at 4°C (Widjojoatmodjo et al, 1991). PCR amplification for *S*. Typhimurium was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, annealing at 63°C for 30 sec, 72°C for 30 sec, and finished with a final extension at 72°C for 3 min and stored at 4°C (Kim et al, 2006). PCR amplification

Table 1. Oligonucleotide primer sets for the detection of *Salmonella* spp.

Species	Primer Sequences (5` to 3`)	Size (bp)	Reference
S. spp.	Replicon P1: TTA TTA GGA TCG CGC CAG GC Replicon P2: AAA GAA TAA CCG TTG TTC AC	163	Widjojoatmodjo et al (1991)
S. Typhimurium	STM4497-f: AAC AAC GGC TCC GGT AAT GA STM4497-r3: TGA CAA ACT CTT GAT TCT GA	310	Kim et al (2006)
S. Choleraesuis and *S*. Paratyphi C	FlinC-F: AAG GAA AAG ATC ATG GCA CAA FlinC-R: GAA CCC ACC ATC AAT AAC TTT G	963	Chiu et al (2005)

for *S.* Choleraesuis and *S.* Paratyphi C were performed with an initial denaturation at 94℃ for 10 min, followed by 35 cycles of 94℃ for 45 sec, annealing at 55℃ for 30 sec, 72℃ for 1 min, and finished with a final extension at 72℃ for 7 min and stored at 4℃ (Chiu et al, 2005). The amplified products were visualized by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed using the colonies from Rambach agar plates by both direct smear and protein extraction methods. For direct smear method, one to two colonies of each isolate were picked using a wooden applicator. The wooden applicator was then smeared over an individual spot on the MSP 96 target plate, creating a confluent layer of bacterial colonies. The bacterial colonies were dried for 5 min, after that 1 µL of matrix solution (α-cyano-4-hydroxycinnamic acid solution; HCCA) was applied to the spot and allowed to dry. This method was performed in twice for each isolate. For protein extraction method, one to two colonies were picked from solid media and inoculated into a 1.5 tube containing 300 µL of deionized water, and then added 600 µL of 100% ethanol. Each tube was centri-

fuged at 16,000×g for 2 min. After discard the supernatant, the pellets were allowed to dry and then were resuspended in 50 µL of 70% formic acid and 50 µL of 100% acetonitrile. The suspension was centrifuged again at 16,000×g for 2 min. Following centrifugation, 2 µL of the resultant supernatant containing the extracted proteins was applied to each well and dried for 5 min. This method was performed in twice for each isolate. MALDI-TOF analysis was performed using the MALDI Microflex LT instrument and Biotyper 3.0 software (Bruker Daltonik, Bremen, Germany). Each 96 target plate was tested using the automated analysis feature of the Biotyper software. Results are given as a score value between 0.000 and 3.000 and the criteria applied for accepting results were shown in Table 2. Confidence scores of less than 1.699 corresponded to "Not reliable identification", scores of 1.700 to 1.999 corresponded to "probable genus identification", scores of 2.000 to 2.299 corresponded to "secure genus identification, probable species identification", and scores of 2.300 to 3.000 corresponded to "highly probable species identification".

RESULTS

Pathology and Microbiology

The pigs with salmonellosis were weaned pigs (39 pigs; 95.1%) ranged from 4 to 12 weeks. These pigs were composed of 25 pigs (61.0%) ranged from 4 to 8 weeks and 14 pigs (34.1%) ranged from 8 to 12 weeks. The other 2 pigs (4.9%) were 25-day-old suckling pig and 130 days old growing pig. Clinically, most pigs showed watery diarrhea (35 pigs; 85.4%) and wasting

Table 2. Meaning of score values by MALDI-TOF MS

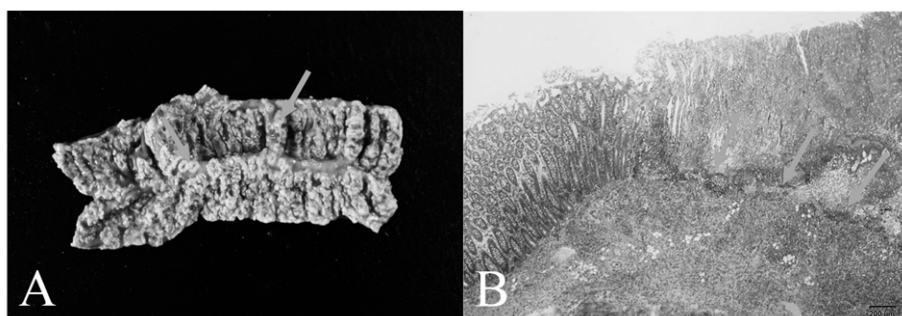| Score Value	Strength of Identification Achieved
2.300～3.000	Highly probable species identification
2.000~2.299	Secure genus identification, probable species identification
1.700～1.999	Probable genus identification
0.000～1.699	Not reliable identification

Fig. 1. Gross and histopathologic findings of large intestine in pig. (A) Note the fibrinous exudates and multifocal to coalescing ulcers (arrows) on colonic mucosa. (B) Note mucosal ulceration (arrows) and submucosal infiltration of inflammatory cells in colon. H&E. Bar = 200 µm.

(31 pigs; 75.6%). Grossly, fibrinous exudate (13 pigs; 31.7%) and ulceration (16 pigs, 39.0%) in the large intestine were prevalent lesions in porcine salmonellosis (Fig. 1A). Histopathologically, variable degrees of enteritis were observed in the large intestine from all pigs. Focal to diffuse, mild to moderate necrosis of intestinal crypt, lamina propria, and surface enterocytes were observed in 9 pigs (22.0%). Multifocal to diffuse ulcerative colitis with/without colitis cystica profunda were frequently observed in 32 pigs (78.0%) (Fig. 1B). Vascular thrombosis composed of fibrin and neutrophils were also frequently presented in the submucosa of large intestine. Among these 32 pigs, *Balantidium coli* were also observed at the ulcerated mucosa or crypts in 9 cases. In bacterial culture, red round colonies were isolated from 41 pigs using Rambach agar plates. And isolated bacteria were confirmed as gram-negative bacilli using Gram staining.

PCR

The results of PCR demonstrated that all 41 isolates were positive for *S.* spp. and 39 isolates (95.1%) were positive for *S.* Typhimurium. However, 2 isolates (4.9%) were all negative for *S.* Typhimurium, *S.* Choleraesuis and *S.* Paratyphi C.

MALDI−TOF MS

MALDI-TOF MS analyses for the isolates were performed both direct smear method and protein extraction method. The level of identification was determined by 0.000 to 3.000 score provided by the Biotyper database. Results for each isolate of *S.* spp. were shown in Table 3.

Using the direct method, 85.4% (35/41) of isolates

Table 3. The number of isolates identified as *Salmonella* spp. by MALDI-TOF MS

Score Value	Direct smear method (n=41)	Extraction method (n=41)
2.300∼3.000	1 (2.4%)	41 (100%)
2.000∼2.299	17 (41.5%)	0 (0)
1.700∼1.999	17 (41.5%)	0 (0)
0.000∼1.699	6 (14.6)	0 (0)

were identified to the genus level and 43.9% (18/41) to the species level. But only one (2.4%) out of 41 isolates was identified to "Highly probable species identification". Surprisingly, all tested 41 isolates (100% to the species level) got score values above 2.300 (high probable species identification) by protein extraction method (Table 3).

DISCUSSION

One of the well-known diagnostic methods for porcine salmonellosis is bacterial identification combined with pathological observations (Griffith et al, 2006). In this study, 41 pigs were diagnosed as *salmonella* enterocolitis based on the gross findings such as watery diarrhea, fibrinous exudates and ulceration in large intestine, and histopathologic evidences in variable degree of necrotic or ulcerative typhlitis and colitis. Furthermore, all red round colonies in selective Rambach agar were identified as *S.* spp. by PCR analysis.

Identification of the causative agents is important for herd management in food animal industries. Manually, identification of *Salmonella* spp. has performed by biochemical, serological and phenotypic analysis after bacterial growth on selective or enrichment media (Saffert et al, 2011). Among the manual bacterial identification procedure, serotyping of *S.* spp. is essential for categorizing *Salmonella* based on the surface antigen differences of somatic (O) and flagellar (H) antigens (Kauffmann-White scheme) (Baggesen et al, 1996; Kim et al, 2006). Although studies have indicated that manual methods are still critical for identification for *S.* spp., it is considered labor-intensive, expensive, complicated, and time-consuming (Abubakar et al, 2007; van Veen et al, 2010; Sparbier et al, 2012). In fact, recent molecular techniques such as real-time (RT) PCR, sequencing analysis, and microarray have introduced for rapid diagnosis of porcine salmonellosis. In particular, PCR methods are highly recommended for bacterial identification since the methods enable to guarantee ease of use, relatively low cost, rapid detection and increased accuracy by amplification of targeted regions from bacterial genome (Kim et al, 2006; Lay, 2001). In this

study, all strains were identified as *Salmonella* spp., though two strains were failed to identify in the genus level.

Recently, MALDI-TOF MS are widely used for accurate and rapid identification of various microorganisms including *Enterobacteriaceae*, non-fermenting bacteria, mycobacteria, anaerobes, and even yeasts (van Veen et al, 2010). This method provides probability of identification at the genus and species levels of microorganisms by measuring of peptide mass fingerprinting from each bacterial species (Table 2) and enable to determine the species of bacteria within few minutes depending on preparation of bacterial protein such as whole cell lysates and bacterial extraction (Mellmann et al, 2008; Sauer and Kliem, 2010; Welker and Moore, 2011). Indeed, a commercial system (MALDI Microflex LT instrument equipped with Biotyper 3.0 software) provides comprehensive, secure databases, and user-friendly software and considered as high-throughput technology for efficient bacterial identification (Emonet et al, 2010).

There are two well-known sample preparation methods for MALDI-TOF MS, which were direct method and extraction method (Anderson et al, 2012). For simple direct method, a single colony can be picked from solid culture media using a swab or toothpick and smeared directly onto a polished steel target plate for identification (Emonet et al, 2010; Anderson et al, 2012). Alternatively in extraction method, isolates require processing through a short formic acid-acetonitrile extraction for 5 min and centrifugation step prior to application on the target (Emonet et al, 2010; Anderson et al, 2012). A study suggested that MALDI-TOF MS guarantee high-throughput and rapid diagnostics at low costs and can be considered as an alternative tool instead of conventional biochemical and molecular identification systems (van Veen et al, 2010).

Previously, identification of *S.* spp. by means of a combination of selective enrichment broth and MALDI-TOF MS was performed using human clinical stool samples (Sparbier et al, 2012). The application of MALDI-TOF MS analysis using the colonies from selective selenite enrichment media from stool samples revealed that a significant number of samples can be iden-

tified with *S.* spp. one day earlier than the standard microbiological procedure. In addition, 60 *S.* spp. isolates from pigs with diarrhea were applied in MALDI-TOF MS based on the direct method and RT PCR, in which the identification rate were 98.3% (59/60) and 100%, respectively (Sohn et al, 2016). However, our study showed that 95.1% (39/41) isolates were confirmed by PCR analysis and 100% (41/41) of isolates were identified to species level of *S.* spp using MALDI-TOF MS based on the protein extraction method. Only 43.9% (18/41) were identified to species level using the direct method. In fact, results suggest that protein extraction method for bacterial isolates enhance the identification rate of bacteria than the direct smear method in MALDI-TOF MS analysis for porcine salmonellosis.

Although applications of MALDI-TOF MS are widely expended, limitations are quite evident. These include several issues such as analyses of uncultivable microorganisms, analyses of samples of mixed strains and differentiation of very closely related taxa (Welker and Moore, 2011). Due to the difficulties with the reproducibility of results by different cultivation conditions between laboratories and the limited of reference spectral sets, MALDI-TOF MS still needs to be improved for species identification of microorganisms (Mellmann et al, 2008). However, with in-depth advances in technology, as well as more accurate methods of sample preparation, some of the current limitations will be overcome in the near future (van Veen et al, 2010; Saffert et al, 2011; Welker and Moore, 2011). Therefore, future applications of MALDI-TOF MS for diagnosis in veterinary medicine will be relied on the expansion of databases such as relevant reference strains of microorganisms for various microbiological fields. In conclusion, the causative agent for porcine salmonellosis can be diagnosed based on the pathological observation, isolation of bacteria by selective media and identification using PCR and MALDI-TOF MS. This study suggests that MALDI-TOF MS is a useful method for salmonellosis in swine industry.

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