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Rapid Detection and Discrimination of the Three *Salmonella* Serotypes, *S.* Pullorum, *S.* Gallinarum and *S.* Enteritidis by PCR-RFLP of ITS and *fli*C Genes

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ABSTRACT *Salmonella enterica* serotype *gallinarum* biovar Gallinarum or Pullorum and *Salmonella enterica* serotype Enteritidis are the most important diseases in poultry industry. Transitional diagnosis methods of these diseases such as direct isolation and identification by a biochemical test are time consuming with low specificity. In this study, we have focused on the suitable procedure for the rapid and accurate diagnosis of diseases derived from the three *Salmonella* strains. We initially confirmed *Salmonella* species by PCR using a specific ITSF/ITSR primer pair instead of biochemical test, and then the PCR-amplified phase 1 flagellin (*fli*C) using a specific *fli*CF/*fli*CR primer pair was digested with a restriction endonuclease, *Bpm* I and/or *Bfa* I, to discriminate among *S.* Pullorum, *S.* Gallinarum, and *S.* Enteritidis. We found that these methods could be applied to field isolates of the three *Salmonella* strains to detect and to discriminate rapidly for convenient diagnosis.

(Key words : *Salmonella*, diagnosis, rapid detection, strain differentiation)

Introduction

Salmonella enterica serotype *gallinarum* biovar Gallinarum or Pullorum and *S. enterica* serotype Enteritidis have been reported to cause major economic problems in poultry industry. *S.* Gallinarum is responsible for fowl typhoid usually found in chicks (Wray et al., 1996; Shivaprasad, 2003), and *S.* Pullorum causes pullorum disease that shows white diarrhea as its major symptom and able to infect older chickens (Wray et al., 1996). Moreover, *S.* Enteritidis can be a significant public health problem and can represents the contamination rate of poultry farms (Rampling et al., 1989; Fadl et al., 1995). Therefore, rapid and correct diagnosis of these diseases is more important in blocking and spreading of the outbreaks. However, unfortunately *Salmonella* strain has the large antigenic diversities over 2,500 serotypes and the conventional methods of the diagnosis require approximately 1 week of diagnosis which includes several steps of growing in selective mediums, conducting biochemical and serological tests or phage typing (Guineess and van Leeuwen, 1978). Moreover differentiation diagnosis between *S.* Gallinarum and *S.* Pullorum responds with low specificity and sensitivity to the conventional methods by showing close relation between biotypes (Cox and Williams, 1976; Ewing, 1986).

In the last years, improved test methods such as restriction fragment length polymorphism (RFLP) of specific genes (Christensen et al., 1992; Kwon et al., 2000), plasmid profiling (Park et al., 1998), ribotyping and pulsed-field gel electrophoresis (Christensen et al., 1992; Olsen et al., 1996) have been reported for the differentiation diagnosis. The purpose of this study is to determine the suitable procedure for the rapid and accurate diagnosis of diseases derived from *Salmonellosis* in poultry industry. Recently, it was published that detected *Salmonellae* with almost serovar using internal transcribed spacer (ITS) region by polymerase chain reaction (PCR) method (Chiu et al., 2005). We applied this method in our first step, colony selection, paralleled with biochemical test to confirm comparison of specificity. Then, specific primers were designed to find the phase 1 flagellin C gene (*fliC*) for the detection to differentiate these three *Salmonella* species which *S.* Gallinarum, *S.* Pullorum, *and S.* Enteritidis by PCR-RFLP with *Bpm* I and/or *Bfa* I.

Materials and Methods

1. Bacterial Strains

Field strains were collected from chicken farms and hat-

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cheries and identified by conventional methods as biochemical typing, API-20E. Further biotying was confirmed through the dulcitol fermentation and ornithine decarboxylation test. The control strain used in this study was donated by the National Veterinary Research and Quarantine Service in Korea. All bacteria glycerol stocks (15%, v/v) were kept at −80 ℃ before experiments.

2. Genomic DNA Extraction

S. Pullorum ATCC 10398-2, *S.* Gallinarum ATCC 9184, *S.* Enteritidis ATCC 13076, *E. coli* DH5α and field-isolated *Salmonella* were cultured with 5 mL of nutrient broth for 18 hrs. Each of 1 mL was subjected to extraction of genomic DNA. Briefly, broth cultures were centrifuged for 5 min at 15,000 rpm and the pellets were resuspended in 100 μL of sterilized distilled water. The samples were boiled at 100 ℃ for 5 min and frozen at −80 ℃ for 5 min in three times and centrifuged for 10 min at 15,000 rpm. The harvested supernatants were applied to amplify target gene as a template.

3. Amplification of ITS Gene

ITS gene primers were designed ITSF (5'-TGCGGCTGGA TCACCTCCTT-3') and ITSR (5'-TATAGCCCCATCGTGTAG TCAGAAC-3') by Chiu et al. (2005). The PCR reaction mixture consisted of 1 μL template DNA and 49 μL solution containing the follows: 5 μL of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl), 1 μL of 2 mM dNTP, 2 μL of ITSF (50 pmol/μL), 2 μL of ITSR (50 pmol/μL), 0.5 μL of Taq polymerase (5 U/μL; Enzynomics, Korea), and 38.5 μL of sterilized distilled water. Amplifications were performed in a 50 μL reaction by using PCR cycler (PTC-220, MJ Research, USA). The cycling conditions were the following: pre-denaturation of 94 ℃ for 5 min, and 35 cycling of 94 ℃ for 30 sec, 60 ℃ for 30 sec, 72 ℃ for 50 sec, and then last extension step at 72 ℃ for 7 min. Ten microliter samples of reaction mixtures were analyzed by gel electrophoresis in 2% agarose gel, dissolved in 1 × TAE (40 mM Tris-Acetate, 1 mM EDTA at pH 8.3) for 60 min at 90V. After staining with ethidium bromide, the amplicons were photographed by Gel Documentation Analyzer (Vilber Lourmat Trance, France).

4. Amplification of *fliC* gene

The primer of *fliC* gene was designed for the differentiation of *S.* Pullorum, *S.* Gallinarum and *S.* Enteritidis. The primers of *fliCF* (5'- GTCGCTGTCCGACAAACATA-3') and *fliCR* (5'-GCAGGAGAGAAATGACGCAA-3') were used to amplify 1,500 bp amplicon. PCR master mixture consisted with 1 μL template DNA and 49 μL solution containing the follows: 5 μL of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl), 1 μL of 2 mM dNTP, 0.25 μL of *fliCF* (50 pmol/μL), 0.25 μL of *fliCR* (50 pmol/μL), 0.5 μL of Taq polymerase (5 U/μL; Enzynomics, Korea), and 42 μL of sterilized distilled water. The cycling conditions by using PCR cycler (PTC-220, MJ Research, USA) were the following: pre-denaturation of 94 ℃ for 5 min, and 35 cycling of 94 ℃ for 30 sec, 60 ℃ for 45 sec, 72℃ for 120 sec, and then last extension step at 72 ℃ for 7 min. 10 μL samples of reaction mixtures were analyzed by gel electrophoresis in 1% agarose gel.

5. RFLP Analysis

The 1,500 bp in size of PCR-amplified *fliC* gene was extracted and purified by Wizard PCR preps DNA purification system (Promega, USA) following the procedures recommended by manufacturer. The 10 μL of purified PCR products were digested and completed for 2 h at 37℃ by a restriction enzyme, *Bpm* I or *Bfa* I (New England BioLab Co., Beverly, MA, USA). Restriction DNA fragment were separated by electrophoresis in 1% agarose gel.

6. Detection of *Salmonella* in Clinical Samples

Clinical samples were obtained from suspected chickens by swabbing liver, spleen, heart, lung and kidney in Chonbuk and Kyeongki provinces in Korea. Those samples were following routine growth procedure and applied PCR for ITS gene, and PCR-RFLP for *fliC* gene, respectively.

Results

1. Rapid Detection of *Salmonella* Species by PCR-amplified ITS Gene

To establish a rapid detection method of field isolates, we initially performed PCR with a pair of primer (ITSF/ITSR) designed from *Salmonellae* specific ITS gene and each of DNA templates extracted from the three standard strains of *Salmo-*

nellae, *S.* Pullorum, *S.* Gallinarum, and *S.* Enteritidis, a field-isolated *S.* Typhimurium, and *E. coli* as a negative control (Fig. 1). As expected, a *Salmonellae* specific 312 bp fragment was amplified in PCR reaction each of the above *Salmonella* strains (lanes 1 to 4) but not appeared in *E. coli* (lane 5). Furthermore, the rapid detection method was applied to a number of field isolates for the discrimination of *Salmonellae* from *Enterobacteriae* (Fig. 2). Prior to subject field isolate to be used in experiments, a number of field-isolated *Salmonella* strains were identified and confirmed as *Salmonella* species by conventional methods using biochemical test. Resultantly, the expected 312 bp fragments were amplified in all of *Salmonella* strains isolated from field samples (lanes 2 to 8) but not appeared in other *Enterobacteriae*, such as *E. coli*, *Klebsiella* species, and *Citrobacter fruendii*, respectively (lanes 9 to 11). These results suggested that PCR amplification of ITS gene could preserve the common strains of the above *Salmonella* strains discussed as in above, which enable the procedure to be conveniently applied to rapid detection and discrimination of *Salmonella* strains from other *Enterobacteria* strains in field conditions.

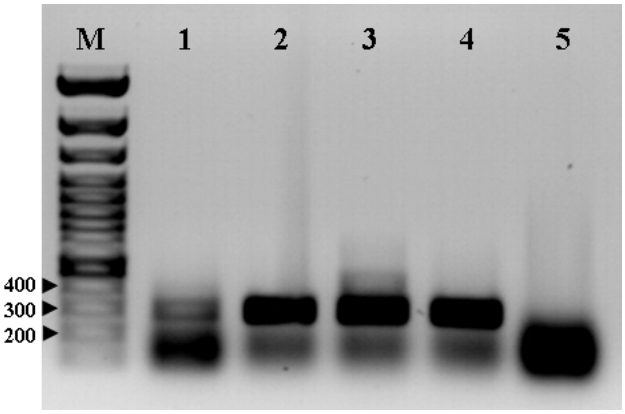

Fig. 1. Specificity of the PCR assay for the detection of *Salmonellae* using primer ITSF/ITSR. M: 100 bp ladder, lane 1: *S.* Pullorum ATCC 10398-2, lane 2: *S.* Gallinarum ATCC 9184, lane 3: *S.* Enteritidis ATCC 13076, lane 4: *S.* Typhimurium from field isolation, lane 5: *E. coli* DH5α strain.

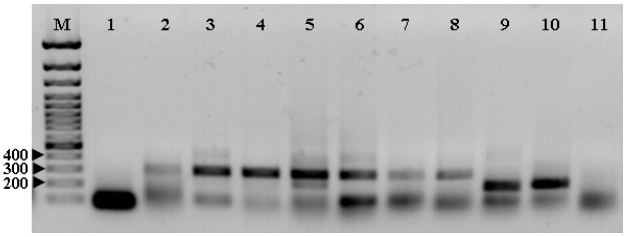

Fig. 2. Applied for the differentiation of *Salmonella* species from *Enterobacteriae* among field isolates. M: 100 bp ladder, lane 1: negative control; sterilized distilled water, lanes 2~4: *S.* Pullorum isolates, lanes 5~6: *S.* Gallinarum isolates, lanes 7~8: *S.* Enteritidis isolates, lane 9: *E. coli*, lane 10: *Klebsiella* species, lane 11: *Citrobacter fruendii*.

2. RCR-RFLP Aassay of *fli*C Genes

The *fli*C PCR system resulted to show a unique amplicon of 1,500 bp both standard and field-isolated strains of *Salmonellae* using a pair of primer (*fli*CF/*fli*CR) designed in the present study. Those PCR products were digested with restriction enzyme(s) *Bpm* I to differentiate *S.* Pullorum from other *Salmonella* strains, and/or *Bfa* I to differentiate *S.* Gallinarum and *S.* Enteritidis from other *Salmonella* strains, respectively (Fig. 3). The digestion patterns of *fli*C amplicon with *Bpm* I conferred a definite distinction among *Salmonellae* including *S.* Gallinarum (lane 2), only *S.* Pullorum given two bands of 572 and 928 bp (lane 1), whereas those with *Bfa* I yielded two bands of 34 and 1,466 bp for *S.* Gallinarum (lane 3) and 572 and 928 bp for *S.* Enteritidis (lane 4), respectively. However, no *Bfa* I site was loca-

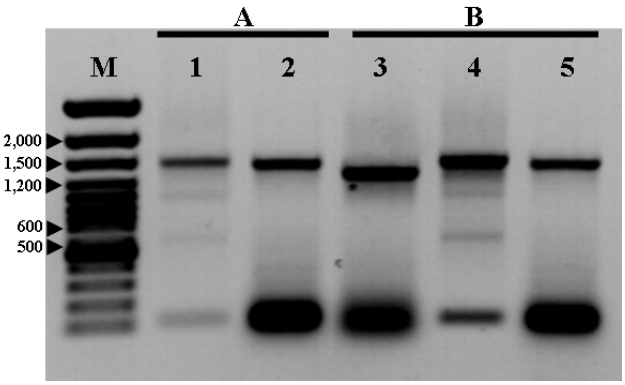

Fig. 3. RFLP assay of PCR-amplified *fli*C gene (A) digestion with *Bpm*I (B) digestion with *Bfa*I from *Salmonellae*. M: 100 bp ladder, lane 1: *S.* Pullorum isolates, lane 2: *S.* Gallinarum isolates, lane 3: *S.* Gallinarum isolates, Lane 4: *S.* Enteritidis isolates, lane 5: *S.* Typhimurium isolates.

ted in *fli*C gene for other *Salmonellae* including *S.* Typhimurium (lane 5). These results suggested that RFLP assays using PCR-apmlified *fli*C genes and two restriction enzymes, *Bpm* I and *Bfa* I, could be conveniently used at least for the strain differentiation among *S.* Pullorum, *S.* Gallinarum, and paratyphoid *Salmonellae*, such as *S.* Enteritidis and *S.* Typhimurium.

Discussion

Although *S.* Gallinarum and *S.* Pullorum can be the serious problems and can cause serious economic losses in poultry industry (Ryll et al., 1996; Shivaprasad, 2003), finding of these diseases by using transitional methods is time consuming and additional tests are closely correlated. Moreover, paratyphoid *Salmonellae*, such as *S.* Enteritidis and *S.* Typhimurium have been the subject of intensified interest as agents of food-borne disease in humans (Rampling et al., 1989; Tauxe, 1991). Advances in poultry production practices, changes in consumer lifestyles and preferences, and heightened nutritional awareness led the poultry products to be the leading source of animal proteins around the world. Contaminated poultry meats and eggs consistently have been among the most frequently implicated sources of human *Salmonella* outbreaks. Therefore, the controlling paratyphoid *Salmonellae* infections in poultry flocks is the important objective from the economic and public health perspectives.

From the perspective of economic losses and control of dissemination of these diseases, it is strongly required to establish a rapid and accurate diagnosis method to minimize the damages. Recently, a number of studies reported to overcome this problem, for example, restriction profiling (Kliger and Grimont, 1993; Dauga et al, 1998), amplify specific genes (Shah et al., 2005), phage typing (Lilleengen, 1952; Guinee and Van Leeuwen, 1978), PCR-RFLP (Kwon et al., 2000; Kisiela et al., 2005) and so on. PCR-RFLP has been applied for the differentiation of *Salmonellae* for objective result, specificity, sensitivity and advantage of saving economic loss and time.

In this study, we confirmed that there is no difference between PCR methods using a primer pair (ITSF/ITSR) and biochemical test for the detection of *Salmonellae* (data not shown). Therefore, we could recommend the method as a convenient approach to detect *Salmonellae* at the first step instead of biochemical test. Furthermore, we analyzed restriction enzyme sites of *fli*C gene with *Salmonella* species to discriminate *S.* Purollum, *S.* Gallinarum, and *S.* Enteritidis based on GenBank database. Digestion of amplified *fli*C gene with *Bpm*I resulted to show 527 and 928 bp only in *S.* Pullorum, and *S.* Derby has *Bpm*I restriction enzyme site but RFLP pattern (339 and 1163 bp) is different from *S.* Purollum. In addition, we could differentiate between *S.* Gallinarum (1,466 and 34 bp) and *S.* Enteritidis (928 and 572 bp) by their RFLP difference that the restriction enzyme sites of *Bfa*I in amplified *fli*C gene exist only in *S.* Gallinarum and *S.* Enteritidis, but not in other *Salmonellae*.

In the present report, we have proposed a diagnostic procedure focused on the major three *Salmonella* diseases in poultry industry, which were mainly caused by *S.* Purollum, *S.* Gallinarum, and *S.* Enteritidis. It is considered to be easily applied as the convenient method of diagnosis according to requirement.

적 요

양계 산업에서 살모넬라에 의한 질병들 중 가장 중요한 원인체는 *S.* Gallinarum, *S.* Purollum, *S.* Enteritidis로 간주된다. 생화학적 검사에 의한 직접적인 균 분리·동정과 같은 이들 질병에 대한 종래의 진단법은 많은 시간이 소요되며, 특이성 또한 낮다. 본 연구는 3종의 살모넬라균에 의해 야기되는 이들 질병에 대한 빠르고 정확한 진단을 위한 효율적인 진단법에 초점을 두었다. 먼저 종래의 생화학적 검사를 대신하여 새로 고안된 ITSF/ITSR PCR primer를 이용하여 살모넬라균임을 확인하였으며, 증폭된 phase 1 flagellin (*fli*C) 유전자를 *Bpm*I 또는 *Bfa*I 제한 효소로 처리하여 *S.* Gallinarum, *S.* Purollum, *S.* Enteritidis를 상호 용이하게 감별하였다. 이상의 결과는 3종의 살모넬라균을 효율적으로 진단하기 위해 신속하게 검출하고 감별할 수 있는 유용한 진단법임을 알 수 있었다.

(색인어 : 살모넬라, 진단, 신속검출, 감별진단)

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