Rapid Detection and Discrimination of the Three *Salmonella* Serotypes, *S.* Pullorum, *S.* Gallinarum and *S.* Enteritidis by PCR-RFLP of ITS and *fliC* 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 ITS/ITSR primer pair instead of biochemical test, and then the PCR-amplified phase 1 flagellin (*fliC*) using a specific *fliCF/fliCR* 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)

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-
cherries and identified by conventional methods as biochemical typing, API-20E. Further biotyping 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 °C 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 °C for 5 min and frozen at −80 °C 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'-TGCGGCTGGAGTCACCTCCTT-3') and ITSR (5'-TATAGCCCCCATCTGTTAGTCAGAAC-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 MgCl2, 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; Enzymomics, Korea), and 42 μL of sterilized distilled water. The cycling conditions were the following: pre-denaturation of 94 °C for 5 min, and 35 cycling of 94 °C for 30 sec, 60 °C for 45 sec, 72 °C for 120 sec, and then last extension step at 72 °C 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 prep 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 °C 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 Salmonella.
nella, S. Pullorum, S. Gallinarum, and S. Enteritidis, a field-isolated S. Typhimurium, and E. coli as a negative control (Fig. 1). As expected, a Salmonella 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 Salmonella 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 freundii, 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 Enterobacteriae strains in field conditions.

2. RCR-RFLP Assay of flic Genes

The flic PCR system resulted to show a unique amplicon of 1,500 bp both standard and field-isolated strains of Salmonella using a pair of primer (flicF/flicR) 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 flic amplicon with Bpm I conferred a definite distinction among Salmonella 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-
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 *Salmonella*, 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 *Salmonella* 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 (Klinger 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 *Salmonella* 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 *Salmonella* (data not shown). Therefore, we could recommend the method as a convenient approach to detect *Salmonella* at the first step instead of biochemical test. Furthermore, we analyzed restriction enzyme sites of *flIC* gene with *Salmonella* species to discriminate *S. Pullorum*, *S. Gallinarum*, and *S. Enteritidis* based on GenBank database. Digestion of amplified *flIC* gene with *Bpm* resulted to show 527 and 928 bp only in *S. Pullorum*, and *S. Derby* has *Bpm* restriction enzyme site but RFLP pattern (339 and 1163 bp) is different from *S. Pullorum*. 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* in amplified *flIC* gene exist only in *S. Gallinarum* and *S. Enteritidis*, but not in other *Salmonella*.

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. Pullorum*, *S. Gallinarum*, and *S. Enteritidis*. It is considered to be easily applied as the convenient method of diagnosis according to requirement.

**References**

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