Differential diagnosis of *Salmonella gallinarum* and *S. pullorum* using PCR-RFLP

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Abstract

Salmonellosis in poultry of Korea is a significant health problem, which causes substantial economic losses. The most common causative agents of chicken salmonellosis are *S. gallinarum* and *S. pullorum*. Traditional methods used to detect *Salmonella* spp. in chicken are tedious, time consuming and confer little guarantee of sensitivity and species specificity. Therefore, a rapid and sensitive method for the differentiation of *Salmonella* serogroup D was assessed. We first amplified the *rfb* genes by PCR and characterized the amplified product by nucleotide sequence analysis. The homology of nucleotide sequence was 99.7% between *S. gallinarum* and *S. pullorum* *rfb* genes. Further comparisons of the sequences of *S. gallinarum*, *S. gallinarum* field strain, *S. pullorum* and *S. typhi* (GenBank Accession No. M29882) showed a homology of 98.3%. The predicted amino acid sequence homology was 97.1%, 97.1% and 97.5%, respectively. Based on this comparison of these nucleotide sequences, we found unique restriction enzyme sites within the *rfb* genes of *S. gallinarum* and *S. pullorum*. Thus, the PCR products could be further digested with restriction enzymes *HfiI* and *PstI* for use in a restriction fragment length polymorphism (RFLP) technique. This method can be applied in the differential diagnosis between *S. gallinarum* and *S. pullorum*.

Key words: *Salmonella gallinarum*, *S. pullorum*, PCR-RFLP, differential diagnosis

Introduction

Fowl typhoid caused by *Salmonella gallinarum* is an economic disease of worldwide significance. It has largely been eradicated from Korea which has an intensive poultry industry but the disease is still an important infectious disease resulting in severe economic distress due to high morbidity and mortality. *Salmonella* infections in humans may manifest in three forms: gastroenteritis, involving an abrupt onset of nausea, fever, vomiting, and diarrhea; enteric fever (typhoid fever), usually caused by *S. typhi*; and septicemia, characterized by fever, chills, anorexia, anemia, and focal lesions on visceral organs [4, 26].

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of *Salmonella* infections. The current standard laboratory procedure to culture and identify *Salmonella* serovars takes approximately 4 to 7 days. In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small numbers of organism. Therefore, a more rapid and sensitive method for identification of *Salmonella* serovars from clinical specimens is needed [4, 11, 26].

For many years the test of choice in diagnosis of pullorum disease and fowl typhoid has been the slide agglutination which was originally developed by Runnels et al. [22] for use with serum and adapted by Schaffer et al. [24] for whole blood by using stained antigen [27]. Recently, other tests such as latex agglutination, enzyme-linked immunosorbent assay and DNA hybridization test have been increasingly applied for the diagnosis. These tests are very rapid but have also been found to suffer from a lack of specificity, a factor which has limited their acceptance [21]. Thus a rapid, unambiguous, specific and accurate method to differentiate *S. pullorum* from *S. gallinarum* has been required.

Recently, polymerase chain reaction (PCR) has been shown to offer a new strategy in the detection of *Salmonella* using *rfb* gene [6, 21, 27, 29, 31]. This PCR
method based on the designed primer sets targeting variable regions of the DNA sequences of the \(rfb\) gene clusters that are involved in biosynthesis of Salmonella lipopolysaccharide (LPS) O antigens [15, 18]. John et al. (1983) [19] reported the use of oligonucleotide primers targeting the aequoase and paratose synthase genes and show that their use in a polymerase chain reaction amplification assay was selective for Salmonella belonging to serogroups A, B, C2, and D. Also, Wyk et al. (1989) [32] identified and sequenced the gene \(rfbJ\) which encodes the final step in the biosynthesis of CDP- aequoase and hence distinguished group B Salmonella from group A and group D Salmonella [13], Verma et al. (1989) [30] have identified, cloned and sequenced \(rfbS\) and \(rfbE\) from group A and group D strains [28, 32]. Within a country, Ahn et al. (1998) [1] reported application of \(rfbS\) gene for specific detection of Salmonella A, D group. Also, Kwon et al. (2000) [16] reported differentiation of Salmonella serogroup by RFLP-PCR based on having polymorphisms.

To date, it has not been reported completely that each Salmonella serotype has or not difference for \(rfb\) gene. And differentiation method for each biotype using \(rfb\) gene has not been developed. Therefore, in this paper we have reported difference of \(rfbS\) gene sequences of \(S.\) gallinarum and \(S.\) pullorum and RFLP method to differentiate these biotypes.

Materials and methods

Bacterial strains

Control strains of Salmonella serotype D group (\(S.\) gallinarum ATCC 9184, \(S.\) pullorum S-11, \(S.\) dublin S-37, \(S.\) enteritidis ATCC 13076, \(S.\) typhi KCTC 2424) were obtained from National Veterinary Research and Quarantine Service in Korea. All of the bacteria were grown in broth Luria-Bertani (LB, Difco Laboratories, Detroit, MI, USA) media, frozen in glycerol and stored at -70°C until needed. Field strains were isolated and identified from poultry farms in Jeonbuk area. The isolates were characterized by using selective media [brilliant green agar, Heiktoen enteric agar, xylose-lysine deoxycholate agar, MacConkey agar, eosin-methylene blue agar (Difco Laboratories, Detroit, MI, USA)] and biochemical methods using API 20E (BioMerieux Vitek, Inc. Anglum Drive Hazelwood, Missouri, USA). Field strains from Jeonbuk, Korea were isolated and identified from liver, lung, trachea, and spleen samples of dead chickens. Following characterizations, the bacteria was grown in LB broth and stored maintained at -70°C until needed.

Experimental design and specimen collection

One-week old chicks were purchased and kept until 110 days age. Experimental infection was done intravenous injection with group A (total 3 chicks) and oral injection with group B (total 3 chicks) receiving \(5 \times 10^5\) CFU (OD600=1) [2, 17]. At the end of the experimental period or at death, specimens collected aseptically from each chicken were liver, lung, intestine, trachea and spleen.

DNA extraction from chicken organs

Two methods were used for DNA extraction from the tissue samples collected from the chickens. In the first, DNaseasy Tissue Kit (Qiagen, Avenue Stanford, Valencia, USA) was used. Twenty five mg of the tissue was homogenized in 1X PBS using a glass homogenizer (Pyrex, USA). Proteinase K (20 mg/ml) was added to each sample, which was incubated for 90 min at 55°C. Then RNase A (100 mg/ml) was added for removal of residual RNA and kept for 2 min at RT. A 200 µl of lysis buffer was added to the homogenate, mixed well and incubated for 10 min at 70°C. Then ethanol was added, mixed well and the mixture loaded onto the DNase mini column. The column was centrifuged at 15,000 rpm for 1 min in a high-speed microcentrifuge. The filtrate was removed, and ethanol from bound DNA was removed with buffer by centrifugation. The DNA was eluted with nuclease free water and was collected by centrifugation at 15,000 rpm for 1min. In the second procedure, InstaGene matrix (BioRad Co, USA) was used for extraction from the bacteria grown from the tissues of the test chickens. Following culture by using nutrient agar from the tissues collected after death, colonies were confirmed by using selective media [brilliant green agar, Heiktoen enteric agar, xylose-lysine deoxycholate agar, MacConkey agar, eosin-methylene blue agar (Difco Laboratories, Detroit, MI, USA)] and biochemical methods using API 20E (BioMerieux Vitek, Inc. Anglum Drive Hazelwood, Missouri, USA). One colony was grown in 5 ml of LB broth media with shaking at 37°C for 12 hrs. Bacterial cells (pellet) with 5 ml LB broth media were collected by centrifugation at 12,000 rpm for 5min, and 100 µl of InstaGene matrix was added. After heating at 56°C for 30 min and at 100°C for 10 min, the supernatant was collected by centrifugation at 15,000 rpm for 1 min.

Oligonucleotide primers and PCR procedure

For PCR analysis of clinical samples, oligonucleotide primers (Bioneer Co., Daejon, Korea) targeting the Salmonella \(rfbS\) gene were used (Table 1) [15]. The 50 µl
mixture contained 5 μl of 10X buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM, MgCl2, 0.001% (w/v) gelatin), 3 μl of dNTPs (2.5 mM), 2 μl of each primer (10 pmol/μl), 0.4 μl of Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), and 5 μl of μl). The reaction was run in a DNA thermal cycler 2400 (Perkin-Elmer Co, Lincoln Centre Drive Foster City, CA, USA) through 30 cycles consisting of: 94°C for 5 min for predenaturatation, 94°C for 1 min (denaturation), 4°C for 30 sec to 1 min (annealing), 72°C for 2 min (polymerization), 72°C for 10 min (post-extension). A 5 μl aliquot of the PCR reaction mixture was electrophoresed on 1.3% agarose gel (Sigma, St Louis, MO, USA) using 100 bp DNA marker (New England BioLabs Co., Beverly, MA, USA) and the separated DNA bands were stained with ethidium bromide (0.5 μg/ml, Sigma, St Louis, MO, USA).

Table 1. Oligonucleotide primers for PCR amplification of a 720 bp product of Salmonella rfbS gene

<table>
<thead>
<tr>
<th>Name of primers (mer)</th>
<th>Nucleotide sequence (5' to 3')</th>
<th>Location product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG1 18 TCAAGACTTACATCTCAGA</td>
<td>40-57</td>
<td>720bp</td>
</tr>
<tr>
<td>SG2 18 CTGCTATATACAGCACAAC</td>
<td>760-743</td>
<td></td>
</tr>
</tbody>
</table>

Direct sequencing of PCR products

QIAquick PCR Purification Kit (Qiagen, Avenue Stanford, Valencia, USA) was used for elution of PCR products for sequencing using PE Biosystems Prism 377 automatic DNA Sequencer (performed by Macrogen in Korea). DNA sequences were analyzed using the GenBank database of the National Center for Biotechnology Information BLAST network service.

And MultiAliin program [23] was used for comparison of nucleotide sequences among Salmonella serotype D group (S. gallinarum, S. pullorum, S. dublin, S. enteritidis, S. typhi (GenBank accession number; GAN M29082)). ALIGN Query using sequence data program was used for percent identity of Salmonella serotype D group. The nucleotide sequence was translated to amino acid sequence using nucleic acid to amino acid translation program which was also used for studying for amino acid homology.

RFLP analysis of the amplified rfbS gene

Based on identified nucleotide sequence of each rfbS gene, restriction enzyme sites were selected from a result by online software for restriction mapping nucleotide sequences (Web cutter, Ver. 2.0). Ten microliters of each purified DNA (0.1 μg/μl) were added to 2 μl of each 10× reaction buffer (New England BioLabs Co., Beverly, MA, USA), 1 μl of restriction enzymes (Tfi I: 5,000 U/ml, Ple I: 1,000 U/ml, New England BioLabs Co., Beverly, MA, USA) and 7 μl of distilled water. After incubation (Tfi I in 1 hr at 65°C, Ple I in 1 hr at 37°C), RFLPs were determined by electrophoresis on 1.3% agarose gels (Sigma, St Louis, MO, USA) using 100 bp DNA marker (New England BioLabs Co., Beverly, MA, USA) and the separated DNA bands were stained with ethidium bromide (0.5 μg/ml, Sigma, St Louis, MO, USA).

Result

Screening for the rfbS gene of serotype D group

With a pair of primers, we are able to amplify a 720 bp DNA fragment of the rfbS gene in the DNA of various Salmonella within the serotype D group (S. gallinarum, S. pullorum, S. dublin, S. enteritidis and S. typhi) and visualize the product by electrophoresis in 1.3% agarose gel (Fig. 1). Also, DNA extracted from specimens collected from poultry farms in Jeonbuk, Korea was amplified from liver, lung, trachea and spleen (Fig. 2).

![Fig. 1. Amplification of rfbS gene from Salmonella species serotype D group by PCR.]

M, 100bp DNA ladder; Lane 1, positive control (S. typhi); Lane 2, negative control; Lane 3, water control; Lane 4, S. gallinarum; Lane 5, S. pullorum; Lane 6, S. enteritidis; Lane 7, S. dublin.

![Fig. 2. Amplification of Salmonella gallinarum rfbS gene from field samples by PCR.]

M, 100bp DNA ladder; Lane 1, positive control (S. typhi); Lane 2, negative control; Lane 3, liver (farm A); Lane 4, liver (farm B); Lane 5, lung (farm A); Lane 6, lung (farm B); Lane 7, trachea (farm A); Lane 8, trachea (farm B); Lane 9, spleen (farm A); Lane 10, spleen (farm B).

Detection of S. gallinarum

The isolates from poultry farms in Jeonbuk area of
Korea were confirmed biochemically to be *S. gallinarum* by isolation on selective media (brilliant green agar, Hektoen enteric agar, xylose-lysine deoxycholate agar, MacConkey agar, eosin-methylene blue agar, Difco Laboratories, Detroit, MI, USA) and biochemical methods using API 20E (Bio-Merieux, Vitex, Inc. Anglim Drive Hazelwood, Missouri, USA). Field isolate was used for experimental infection of chickens (Table 2). After reisolating the organism and confirming it to be *S. gallinarum*, the DNA from various tissues was used for PCR. Group A chickens inoculated intravenously were dead after 2 days, and *S. gallinarum* was detected in the liver, lung, trachea, and spleen. In the small intestine samples, *E. coli* was also detected. Group B chickens, which received the *Salmonella* orally were dead within 7 days (Table 2). *Salmonella* was detected in the liver, lung, trachea, spleen and kidney. As in group A, only *E. coli* was detected in the small intestine. Also, using the DNA from these tissues, *Salmonella rfbS* gene was amplified (Fig. 3).

**Fig. 3.** Detection of *Salmonella gallinarum* rfbS gene from inoculated chickens by PCR. M, 100bp DNA ladder; Lane 1, positive control (*S. typhi*); Lane 2, negative control; Lane 3, liver; Lane 4, lung; Lane 5, trachea; Lane 6, spleen; Lane 7, intestines; Lane 8, kidney.

**Table 2.** Isolation and biochemical identification of bacteria from experimentally inoculated chickens

<table>
<thead>
<tr>
<th>Organs</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 1</td>
<td>No. 2</td>
</tr>
<tr>
<td>Trachea</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Intestine</td>
<td>(±)</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Intravenous injection and death on day 2 post inoculation.*
  
*Oral administration and death within 7 days after inoculation.*

**Nucleotide sequence**

To elucidate the nucleotide differences between two biotypes, each biotype was DNA sequenced, and compared with known sequences from GenBank database (*S. typhi* rfbS gene, GAN M23682). Comparing these sequences with *S. pullorum* rfbS gene, two different oligonucleotide positions were found: 235 bp (oligonucleotide A, *S. gallinarum* and nucleotide G, *S. pullorum*), 596 bp (oligonucleotide A, *S. gallinarum* and nucleotide G, *S. pullorum* S1). And the homology was 99.7% between *S. gallinarum* and *S. pullorum* (Table 3).

**Amino acid sequence**

The homology on the translated amino acid sequences was 100% between the *S. gallinarum* reference strain and the field isolate. When compared with *S. pullorum* and *S. gallinarum* rfbS gene, the homology was 97.1% (one different codon at 200 bp). Also, compared with *S. typhi* rfbS gene (GAN M23682), homology was 97.1% and 97.5% for *S. gallinarum* and *S. pullorum*, respectively (Table 3).

**Enzyme site**

As a result of restriction enzyme sites by Web cutter, three enzymes were found. But unique enzyme sites were found in the nucleotide sequences of rfbS genes of *S. gallinarum* (nucleotide position 235 bp) and *S. pullorum* (nucleotide position 239 bp). These enzymes were *Tfi I* (*S. gallinarum*) and *Ple I* (*S. pullorum*) (Fig. 4). This confirmed the possibility of differentiating between *S. gallinarum* and *S. pullorum*.

**Fig. 4.** Restriction sites from rfbS gene of *S. gallinarum* and *S. pullorum*. A. *S. gallinarum* was digested at nucleotide position 235 by *Tfi I*; B. *S. pullorum* was digested at nucleotide position 239 by *Ple I*.

**Fig. 5.** Digestion product of 235 bp with *Tfi I* but
there was no digestion with *PstI*. *S. pullorum* DNA amplicons gave a digestion product of 239 bp with *PstI*, but was not digested by *TfiI*.

**Table 3. Comparison of nucleotide sequences and translated amino acid sequence of *rfbS* gene among *S. typhi*, *S. pullorum* and *S. gallinarum***

<table>
<thead>
<tr>
<th>Sequence positions</th>
<th><em>S. typhi</em></th>
<th><em>S. pullorum</em></th>
<th><em>S. gallinarum</em> field isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>124</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>237</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>318</td>
<td>A</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>319</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>363</td>
<td>C</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Nucleotide positions</td>
<td>595</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>598</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>600</td>
<td>G</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>624</td>
<td>T</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>669</td>
<td>C</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>686</td>
<td>T</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>687</td>
<td>A</td>
<td>C</td>
<td>C</td>
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<tr>
<td>Translated amino acids</td>
<td>42</td>
<td>L</td>
<td>V</td>
</tr>
<tr>
<td>105</td>
<td>T</td>
<td>Q</td>
<td>Q</td>
</tr>
<tr>
<td>182</td>
<td>S</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>199</td>
<td>E</td>
<td>K</td>
<td>K</td>
</tr>
<tr>
<td>200</td>
<td>A</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>229</td>
<td>V</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

**Discussion**

Differentiation of *S. gallinarum*, *S. pullorum* known as important bacterial pathogens in chickens of Korea has been depended on biochemical tests [3, 9, 10]. Several factors can interfere with the isolation of *Salmonella* serovars from clinical specimens: the condition of the specimen can allow contaminating organisms to inhibit *Salmonella* isolation, antibiotics in infected animals can retard the growth of *Salmonella* organism, or *Salmonella* organisms may be shed only periodically and in low numbers, particularly in carriers [27]. Instead of biochemical tests, PCR and its related method have been reported to identified. Amplification of DNA using PCR can be accomplished rapidly and is of particular value when concentrations of viruses or bacteria are low, when bacteria that are shed are nonviable, or when isolation of an organism is difficult. The PCR can be used as a highly sensitive and specific test for the presence of pathogenic bacteria in clinical specimens [7]. Also, test based on PCR must be more rapid, reliable, and cost-effective than traditional culture methods [25].

The O antigen is highly polymorphic and the variation is thought to be of importance in the pathogenesis of many bacteria including *Salmonella* and the related *E. coli*, with only some antigenic forms being associated with pathogenicity. Many pathogenic *Salmonellae* from groups A, B, or D, have very similar O-antigen structures [13]. Routinely, the *rfb* gene has been for differentiation of *Salmonella* serotype: serogroup A, B, C1, C2, D [13, 15, 19, 28, 30, 32]. Also, among the variety of molecular methods used in genotyping, RFLP-PCR has been commonly used. The RFLP system is inexpensive and easy to perform but requires that a unique set of restriction sites be present in the amplicon of interest [14, 16, 20]. But, it has not been reported completely that each *Salmonella* serogroup has or not difference for *rfb* gene. Also, differentiation method for each biotype using *rfb* gene has not been developed respectively.

Therefore, in this study RFLP-PCR was applied to differentiate the *Salmonella* biotype using the *rfbS* gene with the restriction enzyme. We studied nucleotide sequence of *rfbS* gene to differentiate *S. gallinarum* from *S. pullorum*. From the nucleotide sequence, we identified as follows. The two biotype sequence have been differentiated with known sequences of GenBank database (*S. typhi* *rfbS* gene, GAN M29682). The sequence of gene from isolated from poultry Jeonbuk area (GAN, AP442574) was 100% homologous to the sequence of *rfbS* gene of the
reference strain of *S. gallinarum* (GAN AF442575, ATTC 9184), and this *rfsB* gene sequence had 99.7% homologous to *S. pullorum* *rfsB* gene (GAN AF442575, S11), 98.3% to *S. typhi* *rfsB* (GAN M29682) gene. Also, there was 98.3% homology respectively from the *rfsB* genes of *S. typhi* and *S. pullorum*. In the *rfsB* gene amino acid sequence, when compared with *S. gallinarum* (GBN AF442573) and *S. pullorum* *rfsB* gene (GAN AF442575), homology was 97.1%. Compared with *S. typhi* (GAN M29682) *rfsB* gene, homology was 97.1% and 97.9% in *S. gallinarum* and *S. pullorum*, respectively. Also, from difference of these nucleotide sequences, we confirmed that the unique enzyme site identified with the *rfsB* gene sequence, Tfi for *S. gallinarum* and Pfl for *S. pullorum* could be used in a RFLP method.

In this study, we confirmed that differential method using the *rfsB* gene can be differentiated each *Salmonella* biotype. And these experiment show the possibility of using the RFLP-PCR combination assay for differential diagnosis of other *Salmonella* serogroup species. This report will be first studied using *rfsB* gene for differentiation and investigated difference between *S. gallinarum* and *S. pullorum* *rfsB* gene.

Acknowledgement

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References

18. Liu, D., Verma, N. K., Romana, L. K., and Reeves, P. R. Relationships among the *rfb* regions of *Salmonella*