Standardization of polymerase chain reaction for the detection of *Salmonella gallinarum*

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ABSTRACT

Salmonella is widely distributed in nature. It is present in both cage and litter system poultry farms. Salmonellosis in poultry of Bangladesh causes a marked significant economic loss. The most common causes of chicken Salmonellosis are *S. gallinarum* and *S. pullorum*. Conventional method like isolation and identification to detect *Salmonella* spp. in chicken are tedious, time consuming and confer little guarantee of sensitivity and species specificity. Therefore a rapid, specific and sensitive method for the diagnosis of *Salmonella* is needed. Field samples were collected; bacteria were isolated using standard bacteriological procedure. DNA was extracted using a DNA isolation kit (Wizard® Genomic isolation kit, Promega, USA). The *rfb* gene was amplified by using commercial PCR kit (PCR Master Mixure Kit, Promega®, USA) and genus specific primer as designed elsewhere. The PCR product was subjected for the restriction fragment length polymorphism (RFLP) analysis. A 720 bp product was successfully amplified only from the *S. gallinarum*. RFLP analysis did not show any specific site as previously shown indicating mutation in the restriction site. This method could be applied in the detection and confirmation of *S. gallinarum* from the field samples.

Keywords: *Salmonella gallinarum*, Chickens, PCR, Restriction Fragment Length Polymorphism

INTRODUCTION

Commercial poultry farming is an upgrowing sector started practically during 1980s in Bangladesh (Haque, 2001). Among the different constrains of poultry industries, outbreaks of several devastating diseases is one of the major constraints that causing 30% mortality of chickens per year resulting significant economic loss and discouraging poultry rearing in this country (Das *et al.*, 2005). Salmonellosis is one of the most important bacterial diseases in poultry industry causing heavy economic loss through
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mortality and reduced production (Khan et al., 1998, Begum et al., 1993 and Hoque et al., 1997). Fowl typhoid is frequently referred to as a disease of adult birds caused by Salmonella enterica subspecies enterica serovar gallinarum (Threlfall and Frost, 1990).

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. Even these methods are tedious, time consuming and confer little guarantee of sensitivity and species specificity. In addition, Salmonella serovars are not detectable in certain clinical samples that contain small numbers of organism (Carter et al., 1991, Fricker, 1987, Stone et al., 1994). 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. (1927) for use with serum and adapted by Schaffer et al. for whole blood by using stained antigen (Tuchili et al., 1995). 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.

In consequence a rapid, specific and accurate method to detect salmonellosis in poultry and differentiation of S. pullorum from S. gallinarum is required. PCR is shown to be very unambiguous, highly sensitive, specific and also economically chief to detect Salmonellae. Genus specific PCR has been developed using rfbS gene in some where else (Park, et al., 2001). The present research program has been undertaken for the standarization of PCR for the detection of Salmonella gallinarum based on rfbS gene using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technique has been adoped to differentiate Salmonella gallinarum from S. pullorum.

MATERIALS AND METHODS

Sources of samples

A total of 45 swabs were collected from liver, ovaries and intestine at necropsy from different outbreaks in Bangladesh. All the samples were collected on tetrathionate broth and transferred to Bacteriology laboratory of the department of pathology, BAU, Mymensingh for isolation and identification of Salmonella organisms. Liver, heart, lungs, spleen, intestine and ovary were collected in 10% neutral buffered formalin for histopathological study. Samples were also collected from Central Disease Investigation Laboratory, Dhaka.
Isolation and characterization

Bacteria were isolated and characterized using standard bacteriological and biochemical technique and motility test (Merchant and Packer 1967).

DNA extraction

DNA was extracted from 11 field samples (6 S. gallinarum and 5 S. pullorum) culturing in LB broth (Oxoid Ltd. Bahingstoke, Hamshire, England). Wizard® Genomic DNA Purification Kit (Promegra Corporation. 2800 Woods Hollow Road. Madison, USA) was used to extract the DNA as par the manufacturer instructions. The extracted DNA was quantified using a spectrophotometer’s (Spectronic® Genetics™ New York, USA) expressed in ng/μl.

Amplification of DNA by PCR

The homology of nucleotide sequence was 99.7% between S. gallinarum and S. pullorum rfbS gene. This PCR method based on the designed primer sets targeting variable regions of the DNA sequence of the rfb gene clusters that are involved in biosynthesis of Salmonella lipopolysaccharide capsular proteins (O antigens). One pair of oligonucleotide primer (SG1 and SG2) was used to amplify the rfbS gene of Salmonella that is genus specific to detect Salmonella gallinarum and Salmonella pullorum. Details of the primer can be seen in Table 1. Primers were synthesized by the 1st BASE Pti. Ltd., 41, Science Park Road, Singapore, 117610. SG1& SG2 Primers were diluted with appropriate amount of TE Buffer (PH=8.0) and stored at -20°C until use.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
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<tr>
<td>SG1</td>
<td>5'-tca-cga-ctt-aca-tcc-tac -3'</td>
<td>720 bp</td>
</tr>
<tr>
<td>SG2</td>
<td>5’-ctg-cta-tat-cag-cac-aac-3’</td>
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The rfbS gene was amplified by using commercial PCR kit (PCR Master Mixture Kit, Promega®, USA). PCR reactions were performed on each DNA sample in a 25 μl reaction mixture containing PCR Master Mixture (2x) 12.5 μl, Forward Primer 0.5 μl (100mM), Reverse Primer 0.5 μl (100mM), Nuclease Free water 8.5 μl and Genomic DNA (50ng/μl) 3 μl. 
DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf, Germany) through 30 cycles consisting of 94°C for 5 minutes for predenaturation, 94°C for 60 second (denaturation), 45°C for 60 second (annealing), 72°C for 2 minutes (polymerization), 72°C for 10 minutes (post extension). After completion of cycling program, reactions were held at 4°C. Each experiment was repeated at least three times.

A 5 μl aliquot of the PCR reaction mixture was electrophoresed on 1.3% agarose gel containing ethidium bromide. Agarose gel electrophoresis was conducted in 1X TAE buffer at 100 V for 30 minutes. One molecular weight marker 100 bp DNA ladder size was electrophoresed alongside the PCR products. DNA bands were observed under UV light on a Transilluminator and photographed by Image Documentation System (Labortechnik, Germany).

**Restriction Fragment Length Polymorphism (RFLP)**

In RFLP method two enzymes were used. Tfi I is specific for *Salmonella gallinarum* and Ple I was specific for *Salmonella pullorum*. The DNA amplicon from S. *gallinarum* was given a digestion product of 235 bp with Tfi I but but there was no digestion with Ple I. S. *pullorum* DNA amplicons gave a digestion product of 239 bp with Ple I but, was not digested by Tfi I (Figure 1).

![Figure 1](image-url)
USA) containing ethidium bromide using 100 bp DNA markers. DNA bands were observed under UV light on a Transilluminator and photographed by Image Documentation System (Labortechnik, Germany).

RESULTS

Cultural characteristics and biochemical properties

The salmonella organism was confirmed by both culturally and biochemically. Culturally it showed slight turbidity in tetrathionate broth, colorless colony with a dark central spot in SS agar; colourless, smooth, pale and transparent colonies in MacConkey and pink white opaque colored colonies with brilliant red zone in Brilliant green agar (BGA).

Biochemically the isolated organisms fermented dextrose, mannitol, xylose and maltose and produced either acid and gas or only acid but did not ferment lactose and sucrose. Special emphasis was given on dulcitol fermentation test where S. pullorum did not ferment dulcitol while S. gallinarum fermented dulcitol but produced only acid.

In TSI slant reaction Salmonella organisms produced a red colored slant and yellow colored butt with a blackening precipitate due to H₂S production.

DNA extraction

Overnight culture of LB broth culture of 6 isolates of S. gallinarum and 5 isolates of S. pullorum was used for the DNA extraction. On electrophoresis, the extracted DNA showed good quality without any smear formation. On quantification, DNA yielded high concentration that ranges from 300 ng/μl to 2800 ng/μl.

Amplification of rfbS gene by PCR

The PCR was performed to amplify rfbS gene which is a conserved gene for Salmonella spp. Among 11 isolates 6 S. gallinarum isolates were successfully amplified and gave an amplified product of 720 bp but none of the S. pullorum isolates were amplified (Figure 2).
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Figure 2. Agarose gel electrophoresis of amplified PCR products from rfbS gene using SG1 and SG2 primer pair. Lane1-2: Salmonella pullorum isolates, Lane-3: control condition (no DNA sample); Lane-M: Molecular marker and Lane: 4-8: Salmonella gallinarum isolates.

Restriction fragment length polymorphism (RFLP)

In RFLP method, two enzymes were used. Tfil is specific for Salmonella gallinarum and Plel is specific for Salmonella pullorum. But these enzymes were unable to digest the amplified PCR product (720 bp) of S. gallinarum isolates.

DISCUSSION

The present study was conducted principally to standaradize an effective method for the rapid and sensitive diagnosis of salmonellosis in chickens. In this study 11 samples were used of which 6 were S. gallinarum and 5 were S. pullorum that was identified by standard culture and biochemical methods. The PCR was performed to amplify rfbS gene which is a conserved gene for Salmonella spp. and usually use for the differentiation of Salmonella serotypes, serogroups A, B, C1, C2, D. (Jiang et al., 1991, Kongmuang et al., 1994, Luk et al., 1993, Verma et al., 1988, Widjojoatmodjo et al., 1991, Wyk et al.1989). Our primer pair SG1 and SG2 is able to amplify successfully a 720 bp product from the Salmonella gallinarum isolates but not the S. pullorum isolates. The result coincides with the result of Park et al. (2001) with some differences. Park et al. (2001) was able to amplify the rbf gene of both S. gallinarum and S. pullorum. But in this study only rbf gene of Salmonella gallinarum was amplified but failed to amplify the Salmonella pullorum. From this dissimilarity it may be assumed that this is due to some genetic mutation in the rfbS gene of Salmonella pullorum isolates in Bangladesh.
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 (Kamimura et al., 2001, Kwon et al., 2000, Owen et al., 1999).

The homology of nucleotide sequence between *Salmonella pullorum* and *Salmonella gallinarum* is 99.7% in *rfbS* gene. The similarity among the translated amino acid sequence between *Salmonella pullorum* and *Salmonella gallinarum* *rfbS* gene is 97.1% (Park et al., 2001). Unique enzyme sites were found in the nucleotide sequence of *rfbS* gene of *Salmonella gallinarum* (nucleotide position 235bp) and *Salmonella pullorum* (nucleotide position 239bp). The enzymes were *Tfi I* for *Salmonella gallinarum* and *Ple I* for *Salmonella pullorum* (Park et al., 2001).

The RFLP method was used based on the result of Park, et al. (2001) in Korea where they were able to cut successfully *S. gallinarum* and *S. pullorum* amplicon using *Tfi I* and *Ple I* restriction enzymes. But these enzymes were unable to cut the sequence at the expected site in our isolates. The actual cause of this failure is not clear; but assumed to be some mutation at the enzyme digestion site in Bangladeshi *S. gallinarum* isolates.

However, a 720 bp product was successfully amplified only from the *S. gallinarum*. RFLP analysis did not show any specific site as previously shown indicating mutation in the restriction site. It may further indicate that *rfbS* gene of *S. pullrum* and *S. gallinarum* differ greatly. This method may be applied only in the detection and confirmation of *S. gallinarum*.

**REFERENCE**


PCR for the detection of Salmonella gallinarum


