Standardization of Polymerase Chain Reaction (PCR) for the detection of locally isolated *Salmonella* Pullorum

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ABSTRACT

Pullorum disease is a significant health problem in chickens of Bangladesh. The disease is caused by *Salmonella enterica* subspecies *enterica* serovar Pullorum. Traditional methods used to detect *Salmonella* sp. in chickens is tedious, time consuming and confer little guarantee for sensitivity and species specificity. Genomic DNA was extracted separately from 22 locally isolated *Salmonella* Pullorum from chickens culturing in LB (Luria-Bertani) broth. The using primers 139(F) (5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3') & 141(R) (5'-TCA TCG CAC CGT CAA AGG AAC C-3') for *invA* gene and rfbSP (F) (5'-GAT CGA AAA AAT AGT AGA ATT-3') & rfbSP1.1(R) (5'-GCA TCA AGT GAT GAG ATA ATC-3') for *rfbS* genes were used. DNA was amplified using 139 (F) &141 (R) primer with targeting gene *invA* by PCR methods and found 284-bp amplicon in 1.5 % agarose gel electrophoresis. The target PCR DNA was also amplified using rfbSP (F) & rfbSP1.1(R) primer with targeting gene *rfbS* by PCR methods and 147- bp amplified product did not found at position in 1.5% agarose gel electrophoresis. Among the two genes *invA* and *rfbS*, the primer 139(F) and 141(R) for *invA* gene gave the accurate and sensitive results. Therefore, PCR with 139 (F) and 141 (R) primer for *invA* gene is highly specific as well as sensitive and may be an effective molecular tool in the rapid detection of Pullorum disease in Bangladesh.

Keywords: *Salmonella* Pullorum, Pullorum disease, PCR, Standardization

INTRODUCTION

Pullorum Disease (PD) is one of the most common infectious diseases in chicks in Bangladesh. It is an acute, infectious, and fatal disease of chicks causing much loss during the first 2-3 weeks of age (Chauhan and Roy, 1996). Adult fowls, especially
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laying hens, act as a carrier and transmit infection through eggs to the chicks before hatching (Shivoprasad, 1997). PD is also called bacillary white diarrhoea (Wray and Davies, 2001). PD is caused by the *Salmonella enterica* subspecies *enterica* serovar Pullorum (OIE, 2005). The isolation and identification of salmonellae from clinical samples by traditional cultural techniques are laborious which can last up to 7 days (Stone *et al*, 1994). In recent years, diagnostic laboratories have been concerned with reducing the time required for the diagnosis of *Salmonella* infections and a more rapid and sensitive method for detection and identification of *Salmonella* serovars from clinical specimens is needed. Amplification of DNA sequences unique to an organisms using the PCR improves both the speed of detection and the level of sensitivity at which organisms can be detected (Buffone *et al*, 1991; Ramamurthy *et al*, 1993) and has been increasingly used to identify several bacterial species from food and clinical samples (Stone *et al*, 1994). Another advantage is that PCR is not dependent on the utilization of a substrate or the expression of antigens, thereby circumventing phenotypic variations in biochemical patterns and lack of detectable antigens (Hoorfar *et al*, 1999). The use of PCR for rapid diagnosis is inevitable for the control of Pullorum disease in poultry farms of Bangladesh. Therefore some commercial available primers for specific genes of *Salmonella* Pullorum were amplified and cheeked for sensitivity. The invA gene 284-bp products were more sensitive for locally isolated *S. Pullorum* organisms.

**MATERIALS AND METHODS**

The study was conducted in the Laboratory of the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensing and Livestock Research Institute, Mohakhali, Dhaka, from February 2006 to October 2006.

**Bacteria**

Local isolated *Salmonella* Pullorum received from the Pathology laboratory of Bangladesh Agricultural University, Mymensingh and Livestock Research Institute, Mohakhali, Dhaka.

**Cultivation of *Salmonella Pullorum***

The purity of *Salmonella* Pullorum was verified by cultural, staining and biochemical methods (Carter and Cole, 1990; Cheesbrough, 2000). One colony from each sample was inoculated into 10 ml of LB broth and incubated at 37°C for 18 hours. One ml from each culture was taken into a sterile eppendorf tube for the extraction of genomic DNA.
Oligonucleotide primers

For PCR analysis of locally isolated *Salmonella* Pullorum, oligonucleotide primers (Science Park Rd. #01-23, The Gemini, S’pore) targeting the *Salmonella* *invA* and *rfbS* gene have been used (Table 1).

Table 1. Primers were used for the detection of the *Salmonella* Pullorum

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Length</th>
<th>Primer sequence (5′-3′)</th>
<th>Amplification products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>139(F)</td>
<td><em>invA</em></td>
<td>26</td>
<td>GTGAAATTATCGCCACGTTCCGGCAA</td>
<td></td>
</tr>
<tr>
<td>141(R)</td>
<td><em>invA</em></td>
<td>22</td>
<td>TCATCGCACCGTCAAAGGAACC</td>
<td>284</td>
</tr>
<tr>
<td>rfbSP(F)</td>
<td><em>rfbS</em></td>
<td>21</td>
<td>GATCGAAAAATAGTAGAATT</td>
<td>147</td>
</tr>
<tr>
<td>rfbSP1.1(R)</td>
<td><em>rfbS</em></td>
<td>21</td>
<td>GCATCAAGTGATGAGATAATC</td>
<td></td>
</tr>
</tbody>
</table>

Genomic DNA extraction

Genomic DNA extraction was performed from 22 locally isolated *S.* Pullorum organisms. The method was as follows: 1ml of LB broth overnight cultured with *S.* Pullorum was added to a 1.5ml eppendorf tube. It was centrifuged at 16000 rpm for 2 minutes to pellet the cells and the supernatant was removed. Six hundred µl Nuclei Lysis Solution was added. Gently pipetting was done until the cells were suspended. It was incubated at 80°C for 5 minutes to lyse the cells and then cool to room temperature. Three µl of RNase Solution was added to the cells lysate and inverted the tube 2-5 times to mix. It was incubated at 37°C for 15-60 minutes and then cooled to room temperature. Two hundred rpm for 2 minutes of Protein Precipitation Solution was added to the RNase –treated cell lysate. Then it was vortexed vigorously at 3000 rpm for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. It was incubated the sample on ice for 5 minutes. It was centrifuged at 16000 rpm for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 600µl of isopropanol (37°C). It was mixed gently by inversion until the thread like strands of DNA form a visible mass. It was centrifuged at 16000 rpm for 2 minutes. The supernatant was carefully poured off and drained the tube on clean absorbent paper. Six hundred µl of room temperature 70% ethanol was added. It was centrifuged at 16000 rpm for 2 minutes.
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rpm for 2 minutes and carefully aspirated the ethanol. The tube was drained on clean absorbent paper and allowed the pellet to air dry for 15 minutes. One hundred µl of DNA Rehydration Solution was added to the tube and rehydrated the DNA by incubating at 65°C for 1 hour. Periodically it was mixed the solution by gently tapping the tube. The genomic DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer and 0.8% agarose gel electrophoresis, respectively. Genomic DNA was stored at -20°C freeze until use (Promega, Madison, WI, USA).

**DNA amplification**

PCR amplification was performed in a final volume of 10µl containing 2µl (50ng/µl) of DNA template, Taq buffer- A 1µl, dNTPs 1µl, primer-F 0.5µl, primer-R 0.5µl, Taq DNA polymerase 0.2µl and 4.8µl nuclease free water (Science Park Rd.#01-23 The Geni, S‘pore). Three independent reactions, with the primers, were made for DNA template. Amplification was carried out in Gene amplification PCR system 9600 Thermocycler (eppendorf, Germany), using conditions modified from Doran *et al.* (1996). Initial denaturation was at 94°C for 1min., 94°C for 60 sec., annealing at 50°C for 60 second and extension at 72°C for 21seconds, with a final extension at 72°C for 7 minutes for total 33 cycles and hold for 4°C.

**Agarose gel electrophoresis**

Amplified products were separated by electrophoreses on 1.5 agarose gel containing 5 µg ml⁻¹ ethidium bromide with a 100 bp ladder (Promega, Madison, WI, USA) as molecular weight marker. (Oliveira *et al*, 2003).

**RESULTS**

**Qualitative and quantitative measurement of extracted genomic DNA**

Genomic DNA was extracted from 22 locally isolated *Salmonella* Pullorum samples and then these DNA samples were confirmed by electrophoresis in 0.8% agarose gel for qualitative and quantitative measurement (Figure 1).
Detection of *Salmonella* Pullorum by PCR

Locally isolated 22 field samples of *Salmonella* Pullorum were tested using 139 (F) & 141 (R) primer with targeting gene *invA* and showed 284-bp products from each field samples after 1.5% agarose gel electrophoresis. But control sample did not show any amplicon (Figure 2 and 3). The other set of *Salmonella* Pullorum allele specific primer (rfbSP & rfbSP1.1) did not show 147-bp amplicon after 1.5% agarose gel electrophoresis.
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Figure 2. Electrophoresis on agarose gel showing the 284-bp PCR product (from lane 1 to lane 11) after amplified with invA genes in field samples of locally isolated Salmonella Pullorum, and lane C and lane M showing the negative control and marker, respectively.

Figure 3. Electrophoresis on agarose gel showing the 284-bp PCR product (from lane 12 to lane 22) after amplified with invA genes in field samples of locally isolated Salmonella Pullorum, and lane C and lane M showing the negative control and marker, respectively.
DISCUSSION

Traditionally, diagnosis of Pullorum disease was done by isolation and identification of *Salmonella* Pullorum. The isolation and identification were depended on the culture of the organisms on different selective media and biochemical tests (Haider *et al.*, 2003). Several factors can interfere with the isolation of *Salmonella* serovars from clinical specimens: the condition of the specimen can allow containing 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 carries (Tuchili *et al.*, 1995). Enzyme linked immunosorbent assay (ELISA) test can also be used for the diagnosis of Pullorum disease (Barrow *et al.*, 1992). Instead of biochemical and ELISA tests, PCR and its related method have been reported to identify (Park *et al.*, 2001; Desai *et al.*, 2005). 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 also be used as a highly sensitive and specific test for the presence of pathogenic bacteria in clinical specimens (Cohen *et al.*, 1993). PCR is also more rapid, reliable and cost effective than traditionally culture methods (Carli *et al.*, 2001). We also found the similar results in the present study.

In poultry, control of Pullorum disease depends largely on the identification of the infection in the early stages. Standard bacteriological methods may require 5 to 11 days to isolate and identify *Salmonella* from avian fecal samples. These time-consuming should be complemented with a rapid and primary screening procedure such as PCR (Darwin and Millar, 1999).

Capillary PCR and capillary gel electrophoresis is also sensitive than the conventional PCR methods. This study demonstrates that capillary gel electrophoresis, probably due to the low amount of amplicon with its high detection capacity, aided in visualizing these low amplicons, which could not otherwise be detected with conventional gel electrophoresis. In this investigation, we used *invA* gene and detected precisely the 284-bp specific PCR product analyzed by the Gene Scan 672 software (Carli *et al.*, 2001).

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. The PCR products could be further designed with restriction enzymes *TfiI* and *PleI* for use in a restriction fragment length polymorphism (RFLP) technique. This method can be applied in the differential diagnosis between *Salmonella* Gallinarum and *Salmonella* Pullorum (Park *et al.*, 2001). In the present study,
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the author did not use the restriction enzyme analysis for differentiating the above organisms. Because Salmonella Pullorum was confirmed by biochemical test before PCR.

Rapid differentiation of S. Gallinarum from S. Pullorum could be done by allele-specific PCR. The rfbS allele-specific PCR system was initially applied to the control strain S. Gallinarum and S. Pullorum. S. Gallinarum-specific with primer, rfbSG showed a unique amplicon of 187 bp with the DNA from S. Gallinarum strain but not with S. Pullorum. While, S. Pullorum- specific with primer, rfbSP showed a unique amplicon of 187 bp with the DNA from S. Pullorum strain but not with S. Gallinarum indicating presence of the cited characteristic mutation at position 598 of rfbS gene. S. Gallinarum strains isolated from different tissues samples of clinical outbreaks of fowl typhoid in different geographic location would also have the same characteristic mutation and could thus be rapidly distinguished from S. Pullorum (Shah et al., 2005). An allele-specific PCR assay for the rapid and serotype-specific detection of S. Pullorum has been developed. This method required two steps in which the 720-bp fragment of rfbS gene was amplified, and agarose gel electrophoresis was performed to verify the amplification of desired fragment. This was followed by TfiI and PleI digestion of the PCR amplicons and to identify the polymorphism in the rfbS gene. The designed primers, based on characteristic point mutation at position 237 of rfbS gene, selectively and invariably amplified the 147-bp the amplicon from all isolates. These results also confirm the earlier reports of a polymorphic site located at position 237 of the rfbS gene, which uniformly distinguishes Pullorum (guanine) from non-Pullorum isolates. Pullorum cultures could be achieved in less than 3 hr. This allele-specific PCR assay is rapid and specific, as well as sensitive, and may work out as a novel tool for the rapid and serotype-specific detection of Pullorum in veterinary microbiology or a Salmonella reference laboratory that routinely processes Pullorum disease (Desai et al, 2005). In this investigation, the author also did allele specific PCR using rfbSP (F) and rfbSP1.1(R) primer targeting the gene sfbS of Salmonella Pullorum. The author did not find 147-bp amplicon after 1.5% agarose gel electrophoresis of the PCR products. This may be due small size of amplicon.

Multiplex Polymerase Chain Reaction (mPCR) was developed for the simultaneous detection, in one tube, of Escherichia coli O157:H7, Salmonella spp., Staphylococcus aureus and Listeria monocytogenes using specific primers stx2Af and stx2Ar, IIsf and lIsr, cap8A-Bf and cap8A-Br, Hlyf and Hlyr and showed 553-bp, 405-bp, 312-bp, 210-bp amplicon in 2% agarose gel electrophoresis, respectively. The mPCR method has the additional advantages of a lower economic cost and rapid processing (Park et al, 2006). Multiplex PCR is costly than that of conventional PCR methods. Present study was conducted only for Salmonella Pullorum that is why mPCR is not suggested.
In this study, the *Salmonella* Pullorum organisms was grown in LB broth, extracted DNA and amplified by PCR using invA primer, and found 284-bp amplicon after 1.5% agarose gel electrophoresis. The similar result also found other authors (Olivera et al, 2003; Skyberg et al, 2006). But the author used non-selective and Rappaport-Vassiliadis (RV) selective enrichment broth for the bulk culture of the *Salmonella* Pullorum organisms. Rahman (2006) detected the 50 isolates of *S. enterica* belonging to 11 were found to carry sopB gene irrespective of their serovars like Typhimuriu, Enteritidis, Gallinarum, Choleraesuis, Virchow, etc., and source of isolation. This study demonstrates that the PCR test organisms cultured with LB broth is more sensitive for the detection of *Salmonella* at genus than bacteriological methods.

In conclusion, *Salmonella enterica* serovar *enterica* subspecies Pullorum is a causative agent of Pullorum disease in poultry and is responsible for severe economic losses to the poultry industry in many parts of the world including Bangladesh. A definitive detection of Pullorum requires for the prevention and control of the Pullorum disease.

REFERENCES


