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Evaluation of a Multiplex PCR Assay for Rapid Diagnosis of Fowl Typhoid

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A B S T R A C T

Despite Fowl typhoid (FT) eradication in commercial poultry of some parts of the world, outbreak threats are not eliminated in poultry of developing countries. In the current study, we reported molecular identification of *Salmonella gallinarum* in Indian isolates from backyard poultry by a multiplex PCR assay. Boiled culture lysates of *Salmonella* Gallinarum isolates (*n*=2) from an outbreak showed positive amplifications of *glgC* and *speC* genes in duplex PCR with 174 bp and 252 bp products, respectively. In multiplex PCR assay, primers for salmonella plasmid virulence gene (*spv*C) were also added in same reaction mixture. No amplifications were noticed in negative controls with other *Salmonella* serotypes and non –*Salmonella* organisms. This multiplex PCR assay was found to be rapid and specific in diagnosis of fowl typhoid.

Introduction

Two biovars of *Salmonella enterica* subsp. *enterica* biovar Gallinarum and Pullorum, causing fowl typhoid (FT) and pullorum disease of poultry, respectively are economically important in poultry industry. Eradication in commercial poultry in some parts of the world was achieved through improved surveillance and culling. However, threats of FT outbreaks are not eliminated in commercial and backyard poultry of developing countries (Barrow and Freitas Neto, 2011). Accurate diagnosis of the pathogen is pre- requisite for effective adaptation of control measures. Flock history, mortality, clinical signs and post mortem lesions are suggestive of FT infection but isolation and biochemical identification of the organism still remain 'gold standard' method of confirmation (OIE, 2018). The conventional methods of biochemical identification are laborious, and time consuming. O- and H-antigen specific anti-sera are used commonly for slide and tube agglutination tests to identify Salmonella serovars. Therefore, rapid detection technique of the major *Salmonella* serovars is utmost necessary. DNA based identification of *Salmonella* serovars Gallinarum and Pullorum

was primary interest by many researchers in last few years. Several genes for PCR target in the detection of the serovar Gallinarum were used. Polymorphic areas of *glg*C and *spe*C genes (Kang *et al.,* 2011), flagellar biosynthesis gene *flhB* (Xiong *et al.,* 2016), fimbrial operon gene *bcfD* (Zhuang *et al.,* 2014, fimbrial operon gene *sefA* (Gong *et al.,* 2016), flagellar biosynthesis gene *flhB* (Xiong *et al.,* 2017), SPUL 2693(Xu *et al.,* 2018) were amplified successfully.

Besides, *Salmonella* plasmid virulence (*spvC)* gene is present in the plasmid of seven serovars of *Salmonella* including the most frequent etiologic agents *S. gallinarum-pullorum, S. typhimurium* and *S. enteritidis* (Chiu and Ou, 1996). The *spv* region contains three genes required for the virulence phenotype in mice (Guiney and Fierer, 2011).

In the current study, molecular identification and characterisation was carried out by simultaneous PCR amplification of *glg*C, *spe*C and *spvC* genes with *Salmonella gallinarum* strains from an outbreak of FT in backyard poultry in West Bengal, India.

Materials and Methods

Bacterial strains

Two isolates of *Salmonella gallinarum* (WBSG-1, WBSG-2) obtained from the Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Kolkata, India from an outbreak of FT in Vanaraja fowl were used.

The isolates were conventionally serotyped (antigenic structure 9, 12:-:-) with antisera at National *Salmonella* and *Escherichia* Centre, Kasauli, India. *Salmonella typhimurium* ATCC 13076 and clinical isolates of *E. coli*, *Pseudomonas aeruginosa* were used for negative control in the present study.

Preparation of culture lysate

Bacterial culture lysate was prepared as described previously (Pal *et al.,* 2017). One ml of overnight broth cultures of bacterial growth was taken in 1.5 ml microcentrifuge tube (Tarsons, India) and centrifuged at 6000 rpm for 5 min. The pellet was washed twice with Tris-ethylenediaminetetra acetic acid (EDTA) buffer and was re-suspended in 1 ml Tris-EDTA buffer. Then, the suspension was boiled for 10 min followed by chilling in ice. Then, the supernatant was collected as template DNA after cell debris was removed by centrifugation at 6000 rpm for 5 min and stored at −20°C.

PCR assay

PCR assay was performed by multiplex-PCR targeting *glgC* and *speC* genes in a single reaction mixture as described previously (Kang *et al.,* 2011) with little modifications. The amplification reaction was carried out in a 50 µl PCR mixture containing 10 µl of 5 x PCR buffer, 1.5mM $MgCl_2$, 200 µM dNTPs, 0.6 µM each *glgC* forward and reverse primers, 0.4 µM each *speC* forward and reverse primers respectively, 1.5 U GoTaq Flexi DNA Polymerase (Promega, USA), 5 µl culture lysate DNA and nuclease- free water up to 50 µl. The PCR was carried out in a thermocycler (Eppendorf, Germany) using the following cycle: initial denaturation of 5 minutes at 94 ^{o}C followed by 30 cycles of denaturation each at 94 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 30 seconds, extension at 72 ^{o}C for 30 seconds and final extension at 72 ^{o}C for7 minutes. In triplex PCR assay, primers for *Salmonella* plasmid virulence gene (*spv*C) were also added for detection of virulence plasmid (Table 1).

The electrophoresis was carried out using 2 % agarose gel with 0.5 µl/ml ethidium bromide in 1x TBE buffer at 8V/cm for 1 h. The

amplicons were observed under UV transilluminator (UVP, UK) and photographed.

Results and Discussion

Two boiled cuture lysates of *Salmonella gallinarum* isolates (WBSG1, WBSG2) showed positive amplification of 174 bp and 252 bp in multiplex PCR targeting *glgC* and *speC* genes, respectively (Figure 1). No amplification was observed in negative controls including *Salmonella typhimurium* ATCC 14028. An additional product of *spv*C gene (571 bp) was observed in triplex PCR assay.

Although several PCR assays have been developed for molecular detection of *Salmonella gallinarum*, conventional isolation and biochemical identification of culture is still used as "gold standard". Few PCR techniques were not widely used as they have inherent limitations like requiring additional steps with restriction enzyme digestion (Kwon *et al.*, 2000), semi-nested PCR (Pugliese *et al.*, 2011), or another allele PCR (Shah *et al.*, 2005). However, the primers developed by Kang *et al.*, (2011) correctly identified SG in culture lysates without extraction of pure genomic DNA indicating its usefulness in any diagnostic laboratory and greatly shorten the time of serotype identification. Like our study, Mamnan *et al.*, (2014) also revalidated this duplex PCR assay for investigating outbreaks of fowl typhoid caused by *Salmonella gallinarum* in Kaduna State, Nigeria. This duplex PCR have been reported to differentiate between biovars Gallinarum and Pullorum targeting *glgC* and *speC* genes. Biovar Pullorum does not yield amplicon from *speC* gene using the primers but biovar Gallinarum yields the products of both *speC* and *glgC* genes using these primers (Kang *et al.*, 2011). The *spv* region in virulence plasmid is associated with systemic spread of the pathogen (Heithoff *et al.*, 2008).

Triplex PCR assay in the current study, in addition detects virulence plasmid in the bacterium simultaneously. Xiong *et al.*, (2018) reported another multiplex PCR method focused on three specific genes, *stn*, I137_08605 and *rat*A recently. Based on bioinformatics analysis, they found that gene I137_08605 was present only in *Salmonella pullorum* and *Salmonella gallinarum*, and region of difference in *rat*A gene was deleted only in *S*. Pullorum after comparison with that of *Salmonella gallinarum* and other *Salmonella* serovars.

Table.1 Oligonucleotides (primers) used for multiplex PCR and serovar identification of *Salmonella gallinarum*

Genes	**Primer**	***Oligonucleotides (5' -3')***	**Amplification product (bp)**	**References**
glgC	SG-L	GAT CTG CTG CCA GCT CAA	174	Kang *et al.*, 2011
	SG-R	GCG CCC TTT TCA AAA CAT A		
speC	SGP-L	CGG TGT ACT GCC CGC TAT	252	
	SGP-R	CTG GGC ATT GAC GCA AA		
***spv* C**	SPV-1	ACTCCTTGCACAACCAAATGCGGA	571	Chiu and Ou(1996)
	SPV-2	TGTCTTCTGCATTTCGCCACCATCA		

Fig.1 Multiplex PCR assays for identification of *Salmonella gallinarum*

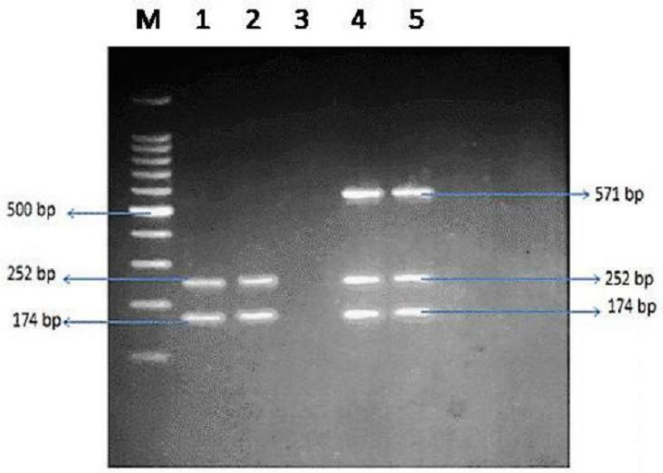

Figure 1: Multiplex PCR assays for identification of of *Salmonella* Gallinarum

Lane M: 100bp DNA ladder, Lane 1: Salmonella Gallinarum(WBSG1), Lane 2: Salmonella Gallinarum (WBSG2), Lane 3: Negative control, Lane 4, Triplex PCR(WBSG1), Lane 5: Triplex PCR (WBSG2)

Triplex PCR based molecular identification has the potential to provide precision in the methods of rapid diagnosis of fowl typhoid in poultry in areas where the disease is enzootic like in India.

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