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Isolation and Detection of Virulence factors of *Salmonella typhimurium* and *Salmonella enteritidis*

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ABSTRACT

Plasmids play an important role in the virulence and antibiotic resistance of *Salmonella*. *Salmonella* carry plasmids having different size and number. Plasmid profile analysis of single isolates of *Salmonella typhimurium* and *Salmonella enteritidis* revealed the presence of 4- plasmid profiles. *Salmonella* serovars produce several Type III secretions including Sop-E and Sop –B. Soap E is detected by Dot-ELISA. Dot-ELISA is one of the solid phase immuneassay used to detect either antigens or antibody. Sop-B (*Salmonella* outer protein-B) is detected by multiplex PCR method using PCR primers *Sop P1* and *SOP- M13*. It is concluded that the technique used in this study is more sensitive and identification and determination of various molecular characteristics of two species enhances the knowledge towards the vaccine production.

1. Introduction

The genus name *Salmonella* has been adopted in the honor of Salmon, who isolated the "hog cholera bacillus" considered to be the causal agent of swine plague (Salmon and smith, 1885). On study of epidemiological feature of salmoellosis, plasmids have been reported to play an important role in virulence and antibiotic resistance of *Salmonella* (Aarestrup et al., 1997).

Salmonella infection is a global problem in human beings and animals. Newborn and young animals commonly suffer from enteric infection within 15 days of their birth (Kaura et al., 2001). *Salmonella* species interact with ileal mucosa and disrupt normal intestinal functions, which result in acute inflammatory cell influx, fluid secretion and enteritis (Wood et al., 1998). Many genes are required for full virulence but only a few of these have been shown to be necessary for the induction of enteritis (Wallis and Galyov, 2000). Another important type III secretion is Sop E, which is associated with invasion by stimulating membrane ruffling (Hard et al., 1998). Production of the enterotoxin (Stn) has been found to be mediated by the presence of Stn and it has been cloned and sequenced (Chary et al., 1993; Chopra et al., 1994). Virulence-associated fimbriae including *Salmonella enteritidis* fimbriae designated as SEF and Plasmid Encoded fimbriae designated as PEF have been identified and cloned (Clouthier et al., 1994). It has been shown that enteropathogenic *Salmonella* strains are able to induce intact intestinal epithelia to

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recruit sub epithelial neuttrophils ((McCormicket al., 1995a, 1995b). *Salmonella dublin, Salmonella tyhimurium* and *Salmonella enteritidis* have been reported to possess large virulence-associated plasmids that range in size from 50 kb to 96 kb (Jones et al., 1998, Popoff et al., 1997). Available data on food poisoning indicate that poultry meat is frequently involved in cases of human salmonellosis (Galbraith, 1990). Bacteria are among the chief causal agents of acute diarrhea and the majority of the bacterial enteropothogens appear to cause fluid lose by stimulation of enterocytes to actively secrete electrolytes (Rao and Field, 1985). Various *Salmonella* serotypes have been shown to produce enterotoxins (Baloda et al., 1983; Rahman et al., 1991a). The gene encoding production of *Salmonella* outer protein (Sop B) is located on *Salmonella* specific DNA fragment representing a pathogenicity island, SPI 5 (Wood et al., 1998). In this study, we used multiplex PCR technique to detect the virulence genes of *Salmonella* species. Dot-ELISA is one of the solid phase immunoassay developed by Hawkes et al., (1982) to detect either antigen or antibody. Panigrahi et al., (1987) developed an immuno-dot-blot assay for detection of cholera related enterotoxin of *Salmonella typhimurium* where he used nitrocellulose paper for absorption of the enteroxin instead of polystyrene plates and they could detect also was 0.02 ng of purified enterotoxin. *Salmonella tyhimurium* and *Salmonella enteritidis* have been reported to possess large virulence-associated plasmids that range in size from 50 kb to 96 kb (Jones et al., 1998, Popoff et al., 1997). Taylor and Wilkins (1961) first observed that strains of *Salmonella* caused fluid accumulation in the rabbit intestinal loops but they left the Cell-Free Culture Supernatant (CFCS) unassayed in the test. Gienella et al., (1974) found that although strains of *Salmonella typhimurium* evoked fluid accumulation observed in the ligated rabbit ileum, their CFCS and its concentrate were unable to induce fluid secretion.

2. Matherials and Methods

2.1. Maintenance of bacterial cultures

Fifty bacterial cultures were collected from the different sources by different methods and maintained through sub-culturing on buffered nutrient agar slants at regular interval of 3 weeks. The cultures were checked for their purity by streaking on Brilliant Agar (BGA) and their morphological, cultural and biochemical characteristics were confirmed (Edwards and Ewing, 1986). All the isolates were identified by biochemical tests.

2.2 plasmid profile

2.2.1. Extraction of plasmid DNA (Alkaline Lysis Method)

Extraction of Plasmid DNA was carried out by alkaline lysis method as per the modified procedure of Kado and Liu (1981) as described by Kinde et al., (1996). The reagents and buffers required for Plasmid extraction are given in appendix. A loop full of culture was grown in 3 ml of Luria broth (Hi-Media) and incubated at 37^{o}C for 16-18 hr. After overnight incubation, the bacteria were harvested by centrifugation at 8000rpm for 10 min. The washed pellet was resuspended in 100µ of (solution I) freshly prepared lysozyme buffer and incubated at room temperature for 10 min. 200µl of (solution II) SDS reagent [0.2 N NaOH, 1% Sodium Dodecyl Sulphate (SDS)] was added to the resuspended bacteria and after gentle shaking, the suspension was left on ice for 10 min. 100µl of (solution III) (pH 5.6) 3 M potassium acetate was added to the suspension. The suspension was then vortexed thoroughly and left on ice for 30 min. The suspension was then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was carefully separated and then mixed with equal volume of phenol: chloroform: isoamyl alcohol solution (25:24:1) and after careful mixing the solution was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was removed and left at room temperature for 5 minutes after thorough mixing. The precipitated DNA was harvested by centrifugation at 15,000rpm for 10 minutes. The DNA pellet was then washed with 70% ethanol to remove the isopropanol and air dried for 15 min. The glassy pellet was then resuspended in TE buffer.

2.3. Agarose gel electrophoresis of plasmid DNA

The plasmid DNA was visualized by carrying out agarose gel electrophoresis in a horizontal submarine electrophoresis (Pharmacia) as the

procedure described by Sambrock et al., (1989). For electrophoresis, 0.7% agarose gel in TBE buffer was used. About 7µl of Plasmid DNA was mixed with 2µl of gel loading buffer as loaded into the gel with the standard markers. Electrophoresis was carried out at 80 V for 2 minutes till the bromophenol blue of the gel loading buffer migrated more than fourth and fifth the length of the gel. At the end of the electrophoresis, the gel was stained with ethidium bromide (0.5mg/ml) and was visualized in the gel documentation system (Image Master-VDS, Amersham Pharmacia, Sweden). Molecular weight of the Plasmid was determined with the help of AAB image software (Amersham Pharmacia, Sweden).

2.4. Detection of Salmonella outer protein (SOP) E

2.4.1. Isolation of Sop E secreted by different strains of Salmonella

The Sop E was isolated from all the strains of *Salmonella* as the method described by Rahman et al., (2001). Bacteria were grown on Luria Bertani (LB) agar (Difco, UK) overnight at 37°C. One colony from agar plate was inoculated in 5 ml of Luria Bertani broth (Difco, UK) containing 0.3 M NaC1 and incubated at 37°C for 6 hour (OD, 0.7 to 0.8) on a rotary shaker (100 rpm). The culture was then diluted 4 times in fresh LB broth (final volume 20 ml) in a 100 ml conical flask and incubated at 37°C for 18 hour on rotary shaker (100 rpm). Then the culture was cooled in ice bath for 30 minute and centrifuged at 20,000rpm at 4°C for 1 hour. The culture supernatant was collected and filtered (0.45µm, Sartorius, Goettingen, Germany). The protein present in the supernatant was precipitated with 10 % (v/v) trichloroaceticacid (Sigma, USA) for 1 hour and then centrifuged at 20,000rpm at 4°C for 1 hour. The sediment was collected and dissolved in 0.4 ml of NaOH (0.1 M) to which 2.0 ml ice-cold acetone (-20°C) was added and incubated at – 20°C for 20 minute. The suspension was centrifuged at 20,000rpm at 4°C for 15 min. The sediments were re-dissolved in 20 ml of acetone (-20°C) and incubated and centrifuged as described above. The sediments were dried at room temperature and dissolved in 0.1ml of PBS and stored at 4°C.

2.5. DOT-ELISA

The Dot-ELISA for detection of Sop E was carried out as described by Rahman (1991) with modification. Standard strains of *Salmonella tyhimurium* and *Salmonella enteritidis* were used as test isolates a single isolate of *E.coli* was used as negative control.

2.6. Assay procedure

Two µl of Sop E preparation of 20 randomly selective strains of *Salmonella* were dotted on polyvinyldine membrane (PVD) strip (Sigma, USA), which was pre-wetted with methanol and dried at 4°C for overnight. The unsaturated sites were blocked by immersing the strips in 3% of bovine serum albumin, fraction V (Sigma) in PBS for 1 hour at 37°C. The strips were washed three times in wash buffer (0.01 M PBS, pH 7.2 with 0.3% Tween-20 and 0.05% Triton X 100) for 5 minutes. The strips were dipped in the Anti-Sop E antisera diluted 1:1000 in wash buffer and incubated at 37°C for 1 hour. After incubation, the strips were washed in wash buffer and incubated with goat anti-rabbit IgG horse radish peroxidise ((HRPO) conjugate (Boehringen, Germany) at a dilution of 1: 1000 for 1 hour at 37°C. The strips were then washed in wash buffer and immersed in freshly prepared substrate solution (sigma fast-TM DAB/ H2O2, Sigma). Washing the strips in running tap water stopped the enzymatic reaction and a position reaction was indicated by the appearance of deep purple dot against a while background within 10-15 seconds.

2.7. Detection of Sop B gene by PCR

The PCR analysis for the detection of Sop B was carried out as per the method described by Rahman (1999). Bacterial calls from boiled at 100°C for 10 minute. After boiling, the cell suspensions were cooled on ice and were immediately tested for Sop B gene by PCR analysis. Primers used for PCR reaction were Sop P1 5-CAA CCG TTC TGG GTA AAC AAG AC-3 (upper primer and) and SOP M13 5-AGG ATT GAG CTC CTC TGG CGAT-3 (lower primer). These primers flank a 397-bp segment in Sop sequence. The PCR mixture (25µl)

included 12.5µl master mix (QIAGEN) containing 2.5 U Tag DNA Polymerase, 200µM each of dATP dCTP dTTP dGTP and PCR buffer, 5µl (1µM) each of upper primer and lower primer and 2.5µl of template DNA (bacterial cell suspension). PCR incubation was performed in a thermocycler (Perkin-Elmer, USA) in 25 cycles of denaturation (94^{o}C for 1 minute), primer annealing (55^{o}C for 1 minute) and primer extension (72^{o}C 2 minute) followed by incubation at 72^{o}C for 10 minute. A 15 µl aliquot of each primer mixture was electophoretically separated on agarose gel (1% containing 0.5 µl/ml ethidiumbromuide, Pharmacia) and the PCR products were visualized in the gel documentation system (Pharmacia).

3. Result

Plasmid profile study carried out for the randomly selected isolates revealed a distinctly varied plasmid profile. Plasmid profile analysis of a single isolate of *Salmonella typhimurium* revealed the presence of 4 different plasmid profiles. And another one only revealed the presence of different plasmid profiles. A single isolate of *Salmonella typhimurium* harbored a 2 different plasmid profiles (Figure 2).

Dot-ELISA test was carried out with sop E preparation of randomly selected 19 isolates in which 2 were *Salmonella typhimurium* and 17 were *Salmonella enteritidis* as test isolates, the *E.coli* was used as negative control. All the 17 test isolates were positive for Sop E, and the *Salmonella typhimurium* showed negative results (Figure 1).

The Sop B gene was found to be present in all 9 positive controls (Table 1) of these strains were found to give rise to 317 bp in the Sop gene segment. It was found to be present in both *Salmonella typhimurium* and *Salmonella enteritidis* isolates and the negative control of *E.coli* 57 was found to be absent for Sop B gene (Figure 2).

4. DISCUSSION

Plasmid profile showed different results based on the species and was found that a single isolate of *Salmonella typhimurium* showed high molecular plasmid. It has been reported that except 2 of the 190 strains were plasmid negative (Mohan et al., 1995). But the present study showed 100% result for the presence of plasmid and their different plasmid profiles also, i. e. a single isolate of *Salmonella typhimutium* showed 4 different plasmid profiles and in *Salmonella enteritidis* also showed 4 different plasmid profiles. It was found that 3, 2 and single plasmid profiles also.

Rahman et al., (1991) examined 29 *Salmonella* strains for the detection of enterotoxin and they found 84.21% of positive result by Dot-ELISA. The present study showed positive result for the presence of Sop E protein in all the *Salmonella enteritidis* isolates and sop E protein is absent in *Salmonella typhimurium* isolates.

Similar type of results were observed by Galyov et al., (1997) observed that sop B (a 60 Kda protein) encoded sop B genes from a sip B mutant of *Salmonella enterica*, and this gene contains 317 bp. The present study confirmed that the Sop B gene contain 317 bp with their results.

5. Conclusion

Plasmid profile showed a multilateral distribution of plasmid amongst both serotypes. A total of 2 plasmid pictures were observed. All the isolates showed different plasmid profiles. Higher plasmid number (three or more) was observed in both isolates.

Detection of sop E was carried out by the immunological assay Dot-ELISA. Twenty isolates were used for this test which 3 isolates belongs to *Salmonella typhimurium* and 17 belongs to *Salmonella enteritidis*. This procedure was very easy and it will be more useful for some other immunological assays.

Detection of Sop B was carried out by PCR using known primers. Sop B, gene detected by the presence of 1348bp product, was found be present in both the isolates of *Salmonella typhimurium* and *Salmonella enteritidis*. Considering its percent detection level, this method was found to be most sensitive. By these detections with PCR amplification technique, it was concluded that technique is more sensitive since its percent detection level.

Identification and determination of various molecular characteristics of two species enhances the knowledge towards the vaccine production.

Table 1. Detection of Virulence genes of *Salmonella typhimurium* and *Salmonella enteritidis*.

S. No	Virulence Genes	No.of *Salmonella typhimurium*/9 isolates	No.of *Salmonella enteritidis*/9 isolates	Molecular Weight
1.	Sop B	4	5	317bp

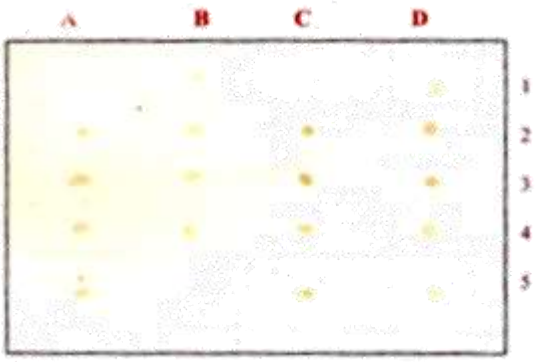

Figure 1. Detection of Sop-E antigen by Dot-ELISA

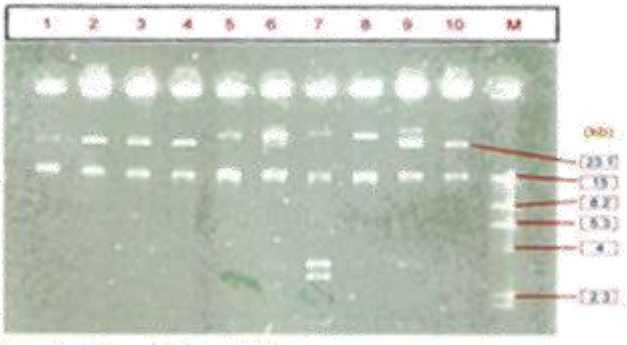

Figure2. Characterization of *Salmonella typhimurium* and *Salmonella enteritidisisolatesfom* man.

Figure 3. Citrate Utilization Test

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