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# Development of Duplex PCR for the Identification of *Shigella* and *Salmonella*: Major Water Borne Bacteria

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**ABSTRACT**

*The present study was focused to develop a duplex PCR for the identification of two major pathogens Shigella and Salmonella from water. A total of 12 waste water samples were collected from sewage treatment plant located in Erode district, Tamilnadu. These water samples were investigated for both microbiological and molecular studies. Standard biochemical test were able to identify tentatively 8 (66.6%) and 11(91.66%) isolates of Shigella and Salmonella respectively. The 23.1Kb genomic DNA were isolated from the bacteria and the protocol was standardized. Duplex polymerase chain reaction was then carried out to identify the presence of the Shigella invasive gene (invC; 875) and Salmonella enterotoxin gene (stn; 617bp). In dPCR out of 12 samples invC gene was detected from 6 (50%) samples andstn gene was detected from 7(58.3%) samples. Virulence gene specific dPCR developed in this study was very sensitive and accurate for the identification of pathogenic strains of Shigella and Salmonella from waste water. In SEM study, the ultra-structure of both the pathogens was visualized. As an outcome of the current study, the virulence genes like invCand stncan be used as a gene marker in PCR assays for the rapid diagnosis of pathogenic strains of Shigella and Salmonella respectively. Present study suggested that PCR is a sensitive, reliable tool over the traditional biochemical tests that are laborious and also time consuming. The study examines the risk of humans exposed to these zoonotic organisms via contaminated water from the sewage treatment plants and may result in serious water-borne illness in humans.*

***Key words:*** *Salmonella, Shigella, sewage water, PCR, virulence.*

## INTRODUCTION

Microbial pathogens in water include viruses, bacteria, and protozoa[1]. Currently, pathogenic bacteria have been identified as the major etiological agent in the majority of the waterborne outbreaks worldwide[2]. Salmonellosis, a disease associated with the gram negative bacterium belonging to the family Enterobacteriaceae affecting both animals and mankind. It is a hyperendemic diarrhoeal disease, caused by a rod shaped, enteric bacteria belonging to genus *Salmonella*[3].

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Similar to the Salmonellosis, bacillary dysentery or Shigellosis is another fatal disease affecting humans and is caused by Gram negative and facultative aerobes belonging to the genus *Shigella* under Enterobacteriaceae family. Theses life threatening fatal diseases usually spread to human via contaminated water. The causative agents of the above two diseases are usually present in untreated sewage water, mixed up with the open or closed drinking water systems and enter into the human once latter consume the water from pond, river and municipal water sources. The virulent strains of these bacteria cause disease posing a serious threat to the public health. Due to the very low infective dose (i.e. <100 bacteria) it is highly transmissible and is spread via the fecal-oral route (i.e. < 100 bacteria)[4]. In the host system *inv* is a virulent genes codes for adhesion and invasion of the pathogen. The *stn* virulence is responsible for enterotoxin production[5]. Hence, accurate and systematic method must be adopted for the screening of virulence genes from *Salmonella* and *Shigella* isolates originated from the infected samples.

The conventional methods established for the identification and detection of the bacterium of the genus *Salmonella* and Shigella are time consuming, low sensitivity and much lesser specificity in interpreting the results, improvement is necessary for the accurate findings[6,7]. Polymerase chain reaction (PCR) has been used by the researchers in recent days for the detection and identification of water borne pathogens by using virulence specific gene detection and found the technique as most reliable and rapid. This molecular tool is catalysed by the enzyme DNA polymerase (Taq polymerase), target DNA fragments are amplified in a chain of replication cycles using oligonucleotide primers[8] is highly specific and sensitive for the identification of microbes and their surveillance[9]. It plays a very successful role in both clinical and environmental samples in detecting and identifying the pathogenic bacteria and applied for examination of these in food and water borne disease outbreaks[10,11,12,13]. Present study is therefore designed to detect *Shigella* and *Salmonella* species by using duplex PCR isolated from sewage water.

## MATERIAL AND METHODS

### Sample details

Twelve outlet water (treated) samples were collected from sewage treatment plant located in Erode district, Tamilnadu. The samples were collected into sterilized container and transported to the Molecular Diagnostic and Bacterial Pathogenomic Research Laboratory of Department of Biotechnology, Bannari Amman Institute of Technology and processed for microbiological investigation.

### Isolation of *Shigella* and *Salmonella*

The water samples were diluted into ten-fold serial dilutions using 2% Buffered Peptone(Difco, USA).Aliquots of 0.1 ml of each dilution tubes were cultured on xylose lysine deoxycholate agar (Difco, USA)[14] by the spread-plate method. Plates were incubated at 37°C for 24 h. Streaking re-streaking were performed in the same agar media to isolate the pure bacterial colonies.

### Identification of *Shigella* and *Salmonella*

Suspected pure colonies were picked from culture plates and subjected to various biochemical assays for the identification of *Shigella* and *Salmonella*.Pure colonies were identified by motility test, Gram and flagellar staining, indole test, methyl red test, Voges-Proskauer test, citrate test (growth on Simmon's citrate agar), urease test, gelatin hydrolysis, $H_2S$ production, acid and gas production test from glucose, mannitol, maltose, sorbitol, adonitol, sucrose, salicin, and lactose. It was found that there were 11 isolates of *Salmonella* and 8 isolates of *Shigella.*

### Ultrastructure study by Scanning electron microscopy

Pure isolates of bacteria were grown on XLD agar plates and were fixed with Karnovsky's fixative (pH 7.3) and incubated at 4°C for 4h. Samples were washed twice with 0.1M Sodium Cocodylate buffer (pH 7.4) (Sigma, USA) and incubated at 4°C for 15 min for each

wash, post fixed with same mix for 12h at 4°C and dehydrated in a series of acetone from 30%-100%, twice in each dehydrating solution for 15 min at 4°C. The samples were dried using the drying reagent tetra methyl silane (Sigma, USA) for 15 min at $4^0$C and air dry in air hood for 15 min. The samples were mounted on aluminium stubs, with adhesives taps and sputter coated with carbon for 5 min using a polaron energy beam and examined under the SEM (Jeol-Jem, Japan).

**Isolation of genomic DNA and agarose gel electrophoresis**

A total of 1.5 ml of bacterial culture was transferred to a 1.5 ml micro centrifuge tube and was centrifuged at 10,000 rpm for 5 minutes. Supernatant was discarded. Pellet was resuspended in 467 ml TE buffer by repeated pipetting.30µl of 10% SDS and 3 µl of Protenase K was added, mixed and was incubated at $50^0$C for 30 minutes. Phenol:Chloroform (1:1) was added and was mixed well by inverting the tube till then the phases were completely mixed.They were then centrifuged at 10,000rpm at $4^0$C for 2 minutes.The upper aqueous phase was transferred to new tubes.$1/10^{th}$ volumes of sodium acetate was added and to that 0.6 volumes of ice cooled iso propanol was added and mixed well by inverting the tubes gently. A white mass of DNA was appeared in the tube.DNA was spooled by means of a glass rod. It is then transferred to a fresh microfuge tube.DNA obtained was washed by adding 1 ml of 70% Ethanol to the tube and centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded and pellet DNA was air dried. The DNA was dissolvedin 50µl TE RNAse buffer by incubating overnight at 4°C. The isolated genomic DNA was stored at $-20^0$C until used. The isolated DNA was run in 1.5% agarose gel, stained with ethidium bromide and visualized in gel documentation system (Biorad, USA).

**Duplex PCR assay**

The duplex PCR assay was developed to detect the virulence encoding genes like invasive gene (*inv*C) and enterotoxin gene (*stn*) of *Shigella* and Salmonella respectively. The forward and reverse primer pairs for *inv*C gene of 875bp were SgenDF1: 5′-tgcccagtttcttcatacgc-3′ and SgenDR1:5′-gaaagtagctcccgaaatgc-3′[15] and *stn* genes of 617bp were For:5′-ttgtgtcgctatcactggcaacc-3′ and Rev:5′-attcgtaacccgctctcgtcc-3′[16] were commercially synthesized (Bangalore Genei, Bangalore). *S.enteric* serovar Typhi (MTCC 733) and *Shigella flexneri* (MTCC 1457)strains were used as positive controls and *Aeromonas hydrophila* (MTCC 646), strain used as a negative control in PCR. The amplification was carried out in 25µL reaction volume containing 12.5µL of 2 × PCR master mix (Promega, USA) containing 4 mM magnesium chloride, 0.4mM of deoxynucleotide triphosphates (dNTPs), 0.5 U of *Taq*DNA polymerase, 150 mMtris-hydrochlroric acid, pH 8.5 (Promega, USA), 1 µM concentration of each forward and reverse primers for *stn*and*inv*C genes, and 2.5µL of template DNA. The PCR reactions were performed in thermal Cycler (Eppendorf, USA). PCR had an initial denaturation for 4 min at $94^0$C and a final extension for 10 min at $72^0$C. The amplification cycles for the PCR undergone denaturation at 94°C for 60 sec, annealing at 59°C for 60 sec and extension at 72°C for 60 sec respectively[5]. The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE (Tris-acetic acid-EDTA, pH 8) buffer, stained with ethidium bromide and observed under gel doc system (BIORAD, USA).

Henceforth, this technique is efficient as it saves the duration and labor by targeting more than one DNA sequence in each reaction. It might not be optimal if the PCR products are limited in certain sizes and agarose gel staining with ethidium bromide[17]. Therefore, it is possible to detect two pathogens in a sample with a single PCR test[18]

## RESULT AND DISCUSSION

In this study, the isolation of *Shigella* and *Salmonella* from 12 outlet water samples of sewage treatment plants was made possible by broth enrichment method. Microbiological investigation revealed the isolation of *Shigella*

8(66.6%) and *Salmonella* 11(91.66%) respectively. The biochemical characteristics of all the isolates of *Shigella* and *Salmonella* were summarized (Figure 1). The isolated Shigella was found to be facultative anaerobic Gram-negative, nonspore-forming, nonmotile, rod-shaped bacteria. The isolated Salmonella was found to be aerobic, Gram-negative, nonspore-forming, predominantly motile, rod-shaped bacteria. Among the biochemical tests, 100% positive results were noticed in Methyl Red test for both *Shigella* and *Salmonella* bacteria, 100% negative results were noticed in Voges-Proskauer and Gelatin hydrolysis tests for the bacteria. Variable results were obtained in tests for indole test, urease and the catalase test of Shigella bacteria citrate, oxidase, catalase and $H_2S$ production for *Salmonella.* In XLD agar, the colonies of *Shigella* were clear to white / pale-redand *Salmonella* were appeared to be light pink colonies with distinct black centres.Similar isolation and identification methods were also adopted by other researchers[19]. In SEM, the *Shigella* and *Salmonella* were appeared to be thick rod shaped (Figure 2).

Genomic DNA was isolated from *Shigella* and *Salmonella* isolates by chemical lysis method. The DNA isolated from these bacteria was electrophoresed and 23.1kb band was visualised in all the isolates.Present result suggested that careful control of the ratio of the volume of each bacterial cells treated to the volume of chemical lysis solution with optimum pH could yield a high quality of chromosomal DNA[20].

Duplex polymerase chain reaction was carried out to identify the presence of the *Shigella* invasive gene (*invC*; 875) and *Salmonella* enterotoxin gene (*stn*; 617bp). In dPCR out of 12 samples *invC* gene was detected from 6 (50%) samples and *stn* gene was detected from 7(58.3%) samples (Figure 3). Virulence gene specific dPCR developed in this study was very sensitive and accurate for the identification of pathogenic strains of *Shigella* and *Salmonella* from waste water. The PCR detection of *stn* gene among the isolates of *Salmonella* also signified the higher risk of food-borne infections caused by these zoonotic bacteria into humans[5]

The persistence of *Salmonella and Shigella* genes in the commercial sewage treatment plant was an important characteristic in its prevalence. Although techniques which in recent years proposed for rapid and reliable detection and confirmation of *Salmonella* are very progress, but the PCR method which we use in our study, yet is a certain technique in identification and confirmation of *Salmonella.* The PCR detection of *inv*C, and *stn* genes among the isolates of *Salmonella and Shigella* also signified the higher risk of water-borne infections caused by these zoonotic bacteria into humans. Therefore, proper treatment of the water is necessary to prevent water brone disease outbreaks in Tamil Nadu India[21].

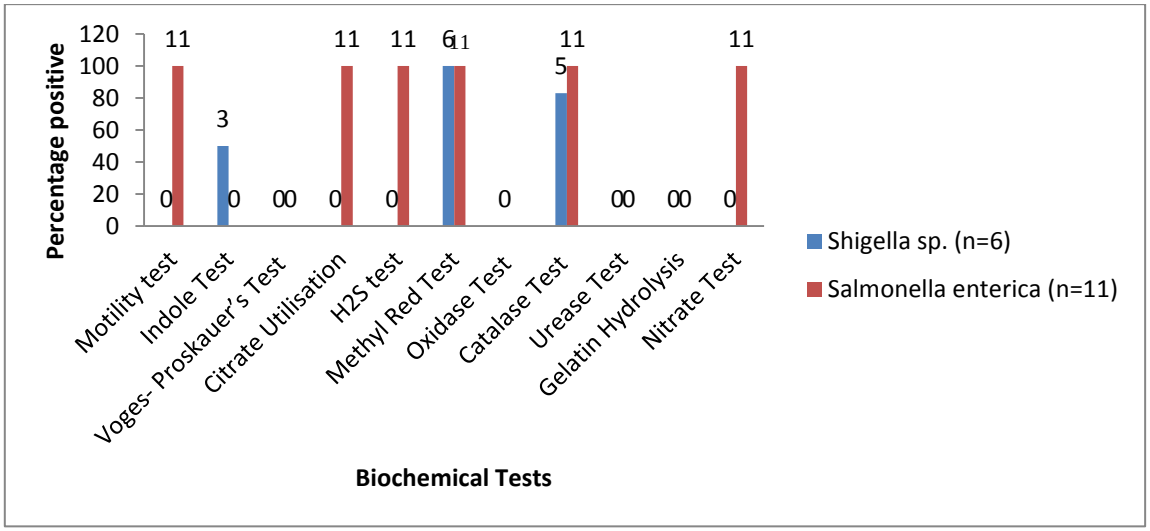


**Fig. 1: Biochemical test of *Shigella* and *Salmonella***

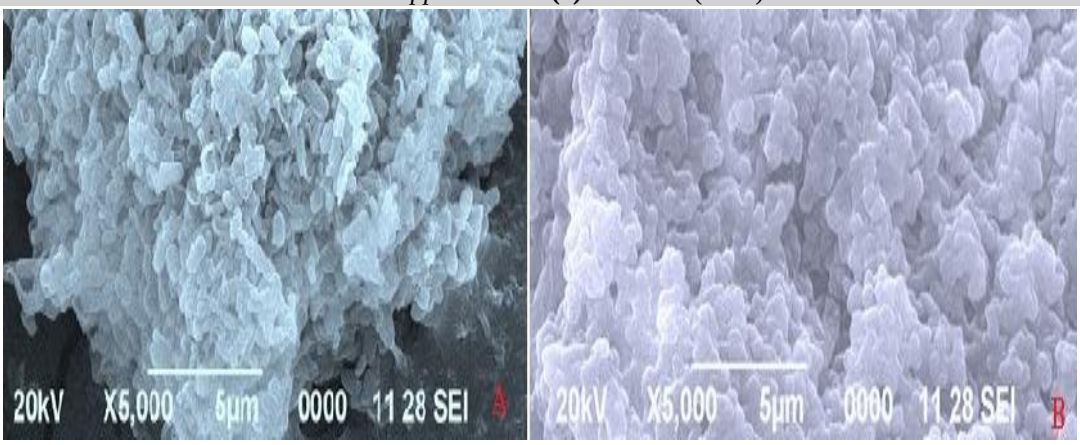


**Fig. 2: SEM analysis of *Shigella and Salmonella* A-*Shigella* and B-*Salmonella***

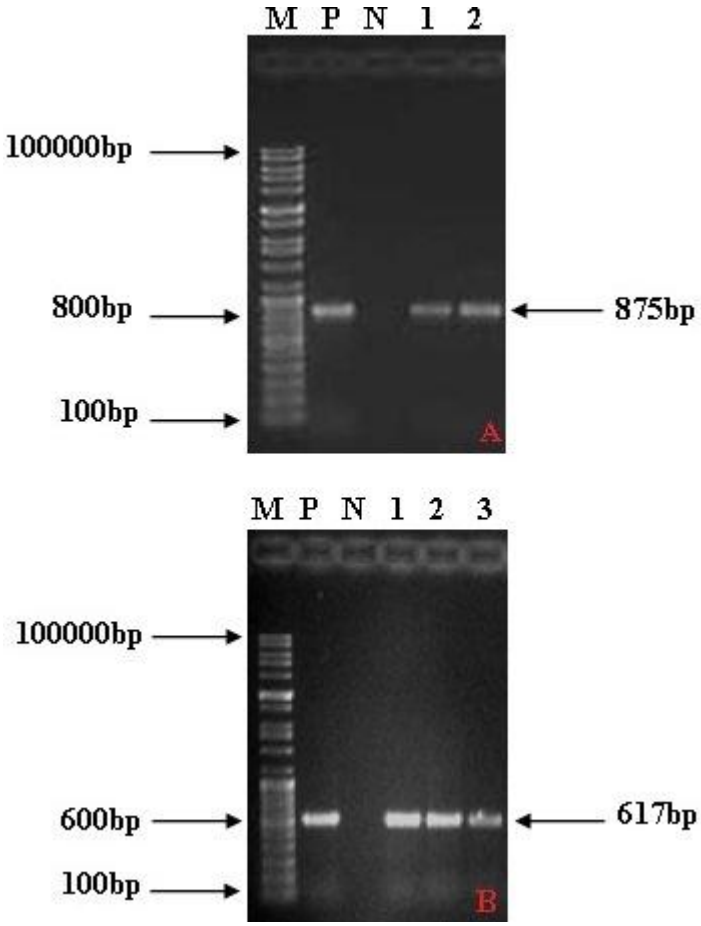


**Fig. 3: PCR based detection of *Shigella* and *Salmonella*.**

A: Detection of *inv*C gene of *Shigella* sp.

B: Detection of *stn* gene of *Salmonella* sp.

M: High range DNA ladder; P: Positive control; N: Negative control; 1,2 & 3: Field isolates

## CONCLUSION

Present study suggested that bacterial pathogens from the environmental sources some times are less in number and such cases, their gene amplification becomes tedious. Hence, enriching the bacteria in an enrichment medium is necessary before performing PCR. PCR is a sensitive, reliable tool for the rapid detection of *Shigella* and *Salmonella* from the commercial sewage treatment plant. High prevalence of virulent strains of *Shigella* and *Salmonella* in the water which is improperly treated from sewage are unsafe and may result in serious water-borne illness in humans. Further, molecular analysis need to be carried out to achieve the accurate distribution of the virulence genes among the various serovars of *Shigella* and *Salmonella* and their protein products in the molecular pathogenesis in humans as well as animals which will help us to develop preventive measures against the deadly pathogens.

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