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MOLECULAR DETECTION OF ENTEROTOXIGENIC ISOLATES OF SALMONELLA TYPHIMURIUM, SHIGELLA FLEXNERI AND STAPHYLOCOCCUS AUREUS

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ABSTRACT

The precise detection of pathogenic organisms that cause food-borne diseases is essential to ensure food safety. The diagnostic procedures should enable to detect enterotoxin producing these pathogens amidst other grown microbial flora and unknown complex food matrix. In the present study, we have developed multiplex PCR for detection of enterotoxigenic food borne pathogens such as *Salmonella typhimurium*, *Shigella flexneri* and *Staphylococcus aureus* by targeting toxin genes such as *stn*, sh*ET* and *seB* respectively. The efficiency of the mPCR method was confirmed with target-positive pure reference culture from spiked milk and bread samples. The detection of these pathogens by mPCR requires at least 36 ng of target-positive DNA from *Staphylococcus aureus*, 360 pg from *Salmonella typhimurium* and as low as 36 pg of DNA from *Shigella flexneri* in the presence of mixture of genomic DNA samples. The mPCR efficiency was evaluated in the presence of single target-positive and several other target-negative *Enterobacteriaceae* family members. This method can be employed as molecular diagnostics method for detection of enterotoxigenic *Salmonella typhimurium*, *Shigella flexneri* and *Staphylococcus aureus* from food samples since mixture of DNAs isolated from spiked food samples provide equivalent efficiency in pathogen detections.

KEYWORDS: Genomic DNA, mPCR, enterotoxin gene, food-borne pathogens.

INTRODUCTION

Microbiological assessment for quality and safety of foods depends upon the specific detection of toxin producing and spoilage microbes. Rapid detection of pathogenic organisms that cause food-borne diseases is essential to ensure food safety. Sometimes it is difficult to

detect enterotoxin producing food-borne pathogens amid large numbers of harmless background microbes in a complex, unknown food matrix with traditional methods.[1] *Staphylococcus aureus*, *Shigella flexneri*, *Escherichia coli,* and *Salmonella typhimurium*are some of the common food-borne pathogens or its enterotoxin frequently found in contaminated food materials. Gutiérrez *et al*[2] noted that *S. aureus* and *Salmonella typhimurium* are main threats to meat industry while *Listeria monocytogenes*, *Bacillus cereus* and *S. aureus* widely found in dairy industry and dairy products. However most of these bacteria can also be found in natural environments. The enterotoxin accumulated in food materials due to presence of these enterotoxigenic bacteria is widely responsible food-poisoning in humans.[3]

However, one of the obstacles to the study of food-borne disease caused by this organism is the differentiation of enterotoxigenic strains from normal flora. *Salmonella* produced enterotoxin through stn gene[4], *Shigella flexneri* produces ShET1 and ShET2-2[5] and *S. aureus* reported to produce around 20 serotypes of enterotoxin and 5 types were reported to be predominant namely SEA through SE. Apart from this enterotoxin, these microbes can secrete some other virulence factors in food materials. However molecular diagnostic methods targeting enterotoxin producing genes can easily differentiate enterotoxigenic food-borne pathogens easier than traditional microbiological methods in food or clinical and environmental samples. Multiplex PCR for two or more food-borne pathogen detections were earlier reported to exist in food industry.[6,7] The few of the PCR based detection of food-borne pathogens were either directly validated in food materials and or after prior enrichment followed by several DNA extraction methods.[6]

In the present study, we developed multiplex PCR systems to detect *S. aureus, Salmonella typhimurium,* and *Shigella flexneri* of Indian origin. For this purpose, enterotoxin target-positive gene stn for *Salmonella*[4], ShET1 and ShET2-2 for *Shigella flexneri*[5] and *SeB* for *S. aureus* species were utilized in PCR systems. Till now more than 31 pathogens have been identified to cause foodborne illnesses in humans and animals (CDC 2011 statistics). However, *Salmonella, Staphylococcus aureus, Shigella flexneri*, *Listeria monocytogenes*, *Clostridium perfringens* and *Escherichia coli* O157:H7 have been generally found to be responsible for the vast majority of illnesses *esp.*, through contaminated foods. In the present study, 3 set of specific primers were combined into single PCR assay to amplify different locus of 3 *Enterobacteriaceae* members. Apparently, the primers were designed to have same

annealing temperature. There may be some interaction between the multiple primer sets, so the primer concentrations may have to be adjusted in order to generate reliable yields of all the PCR products.

MATERIALS AND METHODS

Reference strains and extraction of genomic DNA

Reference strains of target-positive *Staphylococcus aureus* (ATCC-12598 / MTCC- 902), *Salmonella typhimurium* (MTCC-3224) and *Shigella flexneri* (ATCC-29508 / MTCC-1457) along with few other *Enterobacteriaceae* members such as *Bacillus cereus* (ATCC-11778), *Yersinia enterocolitica subsp.enterocolitica* (ATCC-9610), *Staphylococcus epidermidis* (ATCC-155), *Shigella boydii* (ATCC-8700), and *Escherichia coli* (ATCC-37257) were supplied by Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, INDIA. The typical and characteristic red with black centered colonies for *Salmonella typhimurium* and red colonies for *Shigella flexneri* were observed in Xylose lysine deoxycholate agar (XLD Agar). Similarly typical golden yellow colonies were observed for *Staphylococcus aureus* in HiCrome UTI Agar (HiMedia, INDIA) at 37^{o}C for 48-72 hrs. The respective individual colonies were picked and grown in 5 ml of Nutrient Broth (HiMedia, INDIA) for genomic DNA isolation. 2 ml of these pure cultures were taken for DNA extraction using QIAamp Mini kit as per manufacture's recommendation and the isolated DNA was resolved in 0.8% Agarose gel electrophoresis to check the DNA yield and quality.

To determine the minimal amount of target-positive DNAs that would be successfully detected using multiplex PCR (mPCR) assays, the purified DNA molecules of target-positive reference strains was serially diluted after quantification using a UV-Spectrophotometer. Initially, mixture of 3 target-positive genomic DNA samples (3X200 ng) diluted in 10 µl deionsied water to make final template concentration to 60ng/µl. Of which 6 µl was used as template for mPCR, and followed by subsequent serial dilutions (6ng (10^{-1}), 600 pg (10^{-2}), 60 pg (10^{-3}) or 6 pg, (10^{-4}) and 0.6 pg (10^{-5})) ending at 0.6 pg /µl were subsequently used as template for multiplex PCR. Upon completion of PCR, an aliquot (10µl) from each tube was analyzed by electrophoresis. To compare the influencing factors of multiplex procedure, (where mixture of 3 set of primers used) versus that of anyone primer of seB, shET1 and strn with PCR, DNA of the toxin-positive reference strains was tested as a single target versus equal amounts of a mixture of all 5 gene targets.

mPCR detection specificity and Sensitivity assays

To determine the specificity of mPCR, 60 ng of each DNA samples from target toxin-negative species of *S. aureus*, *Salmonella*, *Shigella* and other *Enterobacteriaceae* species such as *Bacillus cereus* (ATCC-11778), *Yersinia enterocolitica subsp.enterocolitica* (ATCC-9610), *Staphylococcus epidermidis* (ATCC-155), *Shigella boydii* (ATCC-8700), and *Escherichia coli* (ATCC-37257) were tested. Sensitivity assays were carried out on spiked egg samples prepared as follows: 0.01 ml of overnight grown individual and mixed cultures of *S. aureus* and *Salmonella, Shigella* were inoculated in eggs and kept for 12-24 hrs. The negative controls were maintained without inoculation. After enrichment, 2 ml of cultures were taken for DNA extraction using QIAamp Mini kit.

RESULTS AND DISCUSSION

Identification of food borne pathogens in food materials is essential to discriminate the contaminated foods from the large and small scale food industry and also to avoid intoxication when ingested by humans. In this study, we presented mPCR method for detection of enterotoxin producing *Salmonella typhimurium, Shigella flexneri* and *Staphylococcus aureus* simultaneously from the mixture of other microbes from contaminated food materials. The mPCR method expected to be cost effective and very accurate for screening of these three target pathogens in any food materials simultaneously.

To optimize mPCR for pathogen detection, different concentration of template and primer concentration were tested to get adequate amplification products in the presence of mixture of target positive DNA. With total template concentration being 360 ng in 20 µl PCR reaction with 3 µl of 20 pmol of mixture of primers produced prominent amplicons for all three pathogens in mPCR as compared to higher concentration of template and primer concentration. The 3 amplicons at expected size of 375 bp (stn), 267 bp (shET) and 88 bp (seB) was clearly resolved in 2.5% Agarose gel electrohporesis with the aid of 50 bp DNA ladder (Fig. 1). The mPCR was tested with one or more target positive DNA, it is able to successfully amplify the given specific target all the time (Fig. 1).

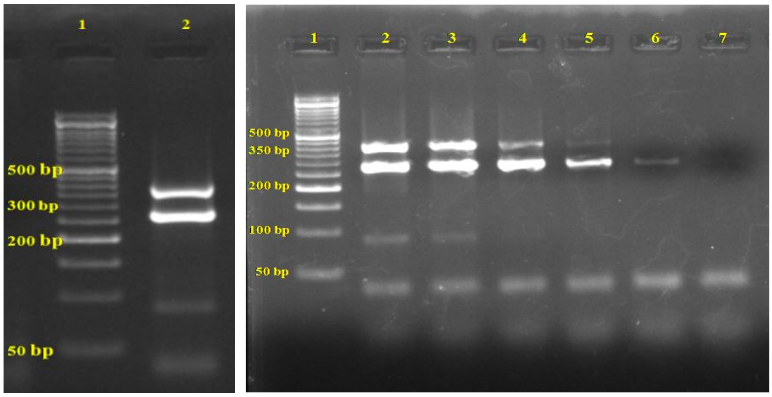

Fig. 1. The mPCR amplicons of stn (375 bp), shET1 (267 bp) and seB (88 bp) of *Salmonella typhimurium* (MTCC-3224), *Shigella flexneri* (ATCC-29508) and *Staphylococcus aureus supsp.aureus* (ATCC-12598) species as resolved by 2.5% Agarose gel electrophoresis (right). The mPCR efficiency of detection of different concentrations (10^{-1} to 10^{-5}) of mixture of genomic DNA of three organisms (left).

Table 1. Primers used in mPCR for the detection of enterotoxigenic Salmonella typhimurium, Shigella flexneri and Staphylococcus aureus strains.

Primer Name	Sequence (5'-3')	Tm (^{0}C)	Product (bp)	Target gene/ Organism
Stn-F	TAC GCT AAT CGT TCC CTG GC	59.4	375	Stn- Salmonella typhimurium
Stn-R	ATT CGT AAC CCG CTC TCG TC	59.4		
SheT1-F	CAC TGT TCA GCG AGG CGT TA	59.4	267	ShET1- Shigella flexneri
SheT-R	CCG GGA ATA TGG ATG TCG CC	61.4		
SeB-F	GTT CGC CTT ATG AGA CTG GCT A	60.3	88	SeB-Staphylococcus aureus
SeB-R	TTT TCA CCA GAT TCA GGC ATC AT	57.1		

mPCR performed with *Salmonella typhimurium* (MTCC-3224), *Shigella flexneri* (ATCC-29508 / MTCC-1457) and *Staphylococcus aureus supsp.aureus* (ATCC-12598 / MTCC- 902) presented amplified regions of stn, shET1 and entB were respectively at expected size (Fig.1). Specificity and sensitivity of mPCR was tested as described in materials and methods section. The mPCR successfully amplified appropriate target regions present in respective species while all other species of Enterobacteriaceae tested in this study were failed to produce amplicons thus indicating there is no chance for cross species binding of primer revealing the target specificity is very accurate. The detection sensitiveness was analysed

using serially diluted mixture of genomic DNA (total concentration of 360 ng comprising all the genomic DNA contributing 60 ng each) from 10^{-1} to 10^{-5} fold as PCR templates. The minimum concentration of genomic DNA is essential for successful mPCR was found to be 60 pg/µl or total concentration of 360 pg for *Salmonella typhimurium*, 36 ng for *S. aureus* and 36 pg for *Shigella flexneri* samples (Fig. 1). In comparison, detection of S.aureus from toxin gene mixture requires higher concentration of template than *Salmonella typhimurium* and *Shigella flexneri.* Therefore to ensure successful application of mPCR for detection of *S. aureus* is possible only if DNA concentration is 36 or more than 36 ng used, hence the overall detection capacity of mPCR set to minimum of 36 ng of each template mixture. In comparison, to single-target PCR, the amplicons intensity of *stn* and *seB* were reduced in mPCR, although same concentration was used in both PCR methods (Fig. 2), though it does not affect the test results. The possible reason would be primer competitiveness and other unknown factors. The mixture of target-positive DNAs ng (6 µl of 60 ng/ µl DNA mixture) isolated from spiked egg cultures of *Salmonella typhimurium, Shigella flexneri* and *Staphylococcus aureus supsp.aureus*, after enrichment, produced equivalent efficiency in mPCR. This efficiency of mPCR would be well utilized for detection of enterotoxigenic food borne pathogens such as *Salmonella typhimurium, Shigella flexneri* and *Staphylococcus aureus supsp.aureus* from other food materials.

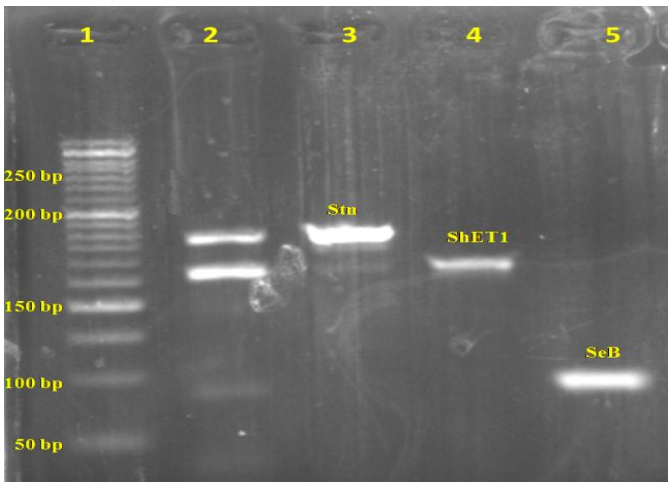

Fig. 2. Evaluation of PCR efficiency with mixture of three toxin target-positive DNA and single target-positive DNA isolated from *S. aureus, Salmonella typhimurium* and *Shigella flexneri;* Lane 1- 50 bp DNA ruler; Lane 2-mPCR with mixture of DNAs from all 3 species; Lane 3-amplicons of *Salmonella typhimurium;*Lan4 and 5 *depicts amplicons of Shigella flexneri* and *S.aureus*

The choice of major diarrheal enterotoxin genes namely *stn*, *shET1* and *seB* to be reasonable as these toxins have been frequently found in food and other clinical samples. For *S. aureus*, *seB* genes were chosen because seB (staphylococcal enterotoxin B) is involved predominantly in food poisoning.[8] In case of *Salmonella typhimurium* enterotoxin coding stn gene was chosen as they encode an etiological agent for *salmonella* mediated food poisoning. Similarly enterotoxin producing gene shET1 of *Shigella flexneri* is responsible for food poisoning. Primers designed for this mPCR were chosen to maintain a near uniform annealing temperature of 61^0C and also care was taken to maintain clear difference between PCR products for better resolution (Table 1). Although multiple factors responsible for pathogenesis of these organisms, the enterotoxin such as shET1, stn and seB and their toxin accumulation in food materials are main etiological agent in respective food poisoning. Stn gene encodes for salmonella heat-labile toxin which had similar biological activities to cholera like toxin (CT-like toxin). Despite the fact that stn being the best studied exotoxins[9], the clear pathway of pathogenesis not clearly understood. Stn prevalent among strains of *Salmonella enterica* but not found in other salmonella species and other *Enterobacteriaceae* species.[5] As evidenced from mPCR in this study, both tested *Salmonella* strains (*Salmonella enterica* (MTCC-3219) and *Salmonella typhimurium* (MTCC-3224) harboring stn gene.

Shigella enterotoxin- shET1 encoded by *set* genes and present in *Shigella flexneri*. As evidenced from our PCR assay, set genes or shET1 toxin gene not present in *Shigella boydii* considered as target-negative strains for mPCR validation. As far as *Staphylococcus aureus* enterotoxin B concerned, encoded by seB toxin gene which is one of the most prevalent in food and clinical samples partly contributing staphylococcal mediated food poisoning cause severe symptoms in humans.[8] Our experiment for enterotoxin gene profiling using PCR with several food isolates also confirmed the high prevalence of *seB* in contaminated food isolates (unpublished data). Therefore this mPCR assay can be effectively utilized to detect most enterotoxigenic isolates of *Salmonella*, *Shigella* and *Staphylococcal* species from food and clinical samples.

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