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Short Communication

Evaluation of a metagenomic detection technique for human enteric bacteria in retail chicken

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The aim of this study was to develop a rapid and reliable protocol for detection of human enteric bacteria in artificially contaminated chicken sample. Tests were performed in inoculated chicken samples using *Salmonella enterica* and *Aeromonas hydrophila*, with dilutions of 10^6,10^5,10^4 CFU/ml. We have developed a direct metagenomic (chicken DNA, inoculated bacterial DNA and endogenous microbial DNA if any) PCR technique for detection of bacteria from this food metagenome. Amplification of respective bacterial 16S rRNA region was performed. PCR conditions were optimized and amplification of *Salmonella enterica* specific DNA was achieved in all samples inoculated with different concentration of bacterial suspension. *Aeromonas hydrophila* infected tissues failed to reveal a specific amplification even after several modifications in gradient PCR. Interestingly, the control (uninoculated) chicken tissues also exhibited a less intense amplification of similar size DNA to target, indicating the possible endogenous contamination of the chicken meat obtained from the retail shop for our analysis.

Keywords: *Salmonella enterica*, *Aeromonas hydrophila,* metagenome, PCR.

Contaminated poultry products are widely accepted as a major source of enteric *Salmonella* and *Aeromonas* infections. Foods of animal origin like fishes and other sea foods, meat and meat products, poultry, eggs, milk and milk products have been reported to be contaminated by these organisms (Arora *et al*., 2006). *Salmonella* and *Aeromonas* have been implicated as potential food poisoning agents and have been responsible for various human infections including gastroenteritis and extra intestinal infections (Porteen *et al*., 2007). One of the most common food borne illnesses Salmonellosis, caused by infection with bacteria from the genus *Salmonella*, is manifested by diarrhoea, mild fever, nausea, and abdominal pains, with the symptoms developing in 12–72 h after consumption of contaminated food.

The traditional methods used to detect *Salmonella* and *Aeromonas* in food, can take 4–7 days to complete as it rely on laborious bacteriological and serological identification. Rapid and accurate detection of *Salmonella* and *Aeromonas*, therefore, continues to be of considerable interest for both food safety surveillance and clinical diagnosis. Among the many rapid methodologies being developed for the detection of *Salmonella* and *Aeromonas* and other food borne pathogens, the polymerase chain reaction (PCR) has been frequently studied over the past decade because, in addition to being rapid and facile, the method can be highly specific and

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sensitive (Chen *et al.*, 2010). However, the direct detection of these bacteria in food sample bye-passing the bacterial and their DNA isolations are still challenging. Thus, the purpose of this study was to establish a PCR protocol for direct metagenomic amplification and detection of *Salmonella* and *Aeromonas* in experimentally infected chicken samples.

Materials and methods

Bacterial strain, growth conditions, and preparation of inoculums

Salmonella enterica and *Aeromonas hydrophilica* (obtained from Institute of Microbial Technology, Chandigarh, India) were grown on Luria Bertani broth at 37°C. After measuring the OD at 600 nm, dilutions were made to obtain 10^6, 10^5, 10^4 CFU/ml.

Artificial inoculation of chicken and sample preparation: Chicken samples were obtained from a local supermarket in Vellore and it was washed with sterile water to remove the exogenous bacteria. Bacterial suspensions at different concentrations in boiling tube were inoculated with approximately one gram of the chicken tissue each and incubated overnight. The tissue samples were washed with several changes of sterile water. The washings were plated on LB agar plates while the tissues were frozen. The plates were observed for bacterial colonies (surface bacteria). The tissues after 12th washing was homogenized in phosphate buffered saline and centrifuged at 12,000 rpm for 10 min. About 30 µl of supernatant was heated at 95°C and centrifuged for 30 sec. The supernatant thus obtained was used as DNA sample in PCR.

PCR primer design

Whole genome sequences of *Aeromonas hydrophila* sub sp. *hydrophila* ATCC7966, *Salmonella enterica* sub sp. *enterica* serovar Newport str.SL254 were retrieved from NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). The sequences of first 16S rRNA were chosen. Primers specific to each species was designed manually, every time performing BLAST search to confirm the species specificity. The sequence of species specific primers is given in Table 1. The primers were synthesized at Priority Life Sciences Private Limited, Hyderabad, India.

Table 1: Primers used in the study for the amplification of 16S rRNA coding region

Primer ID	Bacteria	Sequence (5′ to 3′)
SBSal1F	*Salmonella enterica*	TCA TGG CTC AGA TTG AAC GC
SBSal1R	*Salmonella enterica*	CAT TGT AGC ACG TGT GTA GC
SBAer1F	*Aeromonas hydrophila*	CAG AAG AAG CAC CGG CTA ACT C
SBAer1R	*Aeromonas hydrophila*	TTA CCT TGT TAC GAC TTC ACC

PCR analysis

PCR amplification of the 16S rRNA coding region of *Salmonella enterica* and *Aeromonas hydrophilica* was carried out with metagenomic DNA as templates. The PCR mixture contained 2 µl of metagenomic DNA (obtained as supernatant after boiling the chicken tissue homogenate), 0.2 µM each of forward and reverse primers, 10 µl of master mix (Genei, Bangalore, India) and 5 µl of nuclease free water in a final volume of 20 µl. Amplification was carried out in a thermal cycler (Applied Biosystems, USA) and the reaction mixture was subjected to the following thermal cycling conditions: initial denaturation at 95°C for 2 min; 30 cycles with heat denaturation at 95°C for 1 min, primer annealing at 54°C for 1 min, and DNA extension at 72°C for 1 min. Annealing temperature for *Aeromonas hydrophila* was 52°C, while all other conditions remained same. After the last cycle, samples were maintained at 72°C for 10 min to complete synthesis of all strands. The amplified products were separated in 1% agarose gel with molecular weight marker in one lane.

Results and Discussion

Metagenomic DNA obtained from chicken infected with *S. enterica* resulted in 1.2 kb amplified product (Fig. 1) which is of the expected size. However, metagenomic DNA obtained from chicken infected with *A. hydrophila* failed to result in amplification of the bacterial 16S rRNA coding sequence.

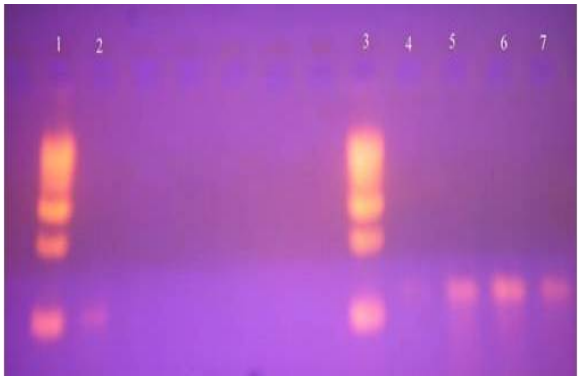

Fig. 1. PCR detection of *Salmonella enterica*, *Aeromonas hydrophila* in chicken metagenome Lanes 1 - 1 kb DNA ladder; 2 – control for *Aeromonas hydrophila* ; 3 – 1 kb DNA ladder ;4- control for *Salmonella enterica*; 5 -10^6 CFU ; 6 - 10^5 CFU; 7 - 10^4 CFU).

A control was maintained in which homogenate was obtained from tissues that are not inoculated with bacteria. At all the dilutions tested, *Salmonella enterica* 16S rRNA target region was amplified from internal tissues of chicken meat. The intensity of the amplified band decreased with decrease in the CFU indicating the sensitivity of the detection procedure. *Aeromonas hydrophila* infected tissues failed to reveal a specific amplification even after several modifications in gradient PCR. Interestingly, the control (uninoculated) chicken tissues in both cases exhibited a less intense amplification of similar size DNA to target, indicating the possible endogenous contamination of the chicken meat obtained from the retail shop.

Salmonella can enter the food chain at every stage, and the consequences for humans after consumption of the contaminated product depend on the food processing conditions. One of the well known sources of contamination is the environment of slaughterhouses for incoming non-affected animals. Later *Salmonella* can multiply to harmful levels due to inappropriate storage conditions. Generally *Salmonella* does not grow at temperatures below 6°C for as long as 15 days on chicken meat, while significant growth has been reported at 8°C.

Salmonella diagnosis is generally performed by standard culture microbiology methods from 25 g of food products. However these methods are time consuming and require costly handling and storage of food during testing. Commercialized analytical techniques are more rapid but not very sensitive. The most probable number (MPN) test is particularly used to determination of low concentration of *Salmonella*. PCR has been suggested as a tool for pathogenic organism detection (Abubakar *et al.*, 2007). However, direct application of PCR to complex substances has been reported to results in no amplification products (Li *et al.*, 2000) or poor sensitivity (Fluit *et al.*, 1993). To circumvent this issue, we have developed a simple and sensitive procedure for direct PCR amplification of bacterial target DNA from the food sample. We have proved that *Salmonella* could be detected to as low as 10^4 CFU concentrations in the infected meat tissue. However, further validation of the procedure with much lower concentration of bacteria is needed for practical use of this technique in large scale meat processing industries.

In a study conducted by Suresh *et al.* (2005) on the incidence of *S. enteritidis* in different sources of retail chicken outlets, cutting board was suggested to be the most common source for the incidence of this organism. The observation of *Salmonella* and *Aeromonas* in control samples (as evident from

expected size amplicons in PCR) is supported by this previous observation. The endogenous presence of these bacteria in the samples used for our study might have occurred at the chicken outlet through cutting board contamination.

Although chicken is usually not consumed raw, there is risk of enteric bacterial infection if the chicken is improperly cooked, and there is also the possibility of cross contamination of *Salmonella* with foods that are consumed raw. Therefore, the ability to rapidly detect *Salmonella* in chicken and other foods could lower the risk of contaminated food reaching the consumer. In accordance with this aforesaid fact, our metagenomic detection procedure represents a valid technique for rapid detection of human enteric bacteria in chicken meat.

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