

Polymerase chain reaction (PCR) for the rapid detection of *Salmonella* in desiccated coconut (DC)

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Abstract

This study was carried out to develop a polymerase chain reaction (PCR) protocol for the detection of *Salmonella* in desiccated coconut (DC). For the PCR detection of *Salmonella*, genomic DNA was extracted using the 'boiling lyses' method and the reaction was carried out with *Salmonella* genus specific primers enabled to amplify 457bp sequence covering *invA* and *invE* genes. Samples of DC produced in mills already tested for *Salmonella* using conventional cultural methods gave identical results with the present PCR method indicating its suitability for adoption in routine testing.

The sensitivity checked using DNA extracted from artificially inoculated DC with serially diluted inoculum of *Salmonella* M1 type showed that the developed PCR method can be used to detect very low levels of contamination of *Salmonella* as low as 4 CFU/g in DC.

The method described here reduces the testing and detection time from 6 days to 24 hours ensuring exporters to obtain *Salmonella* test reports just prior to shipment.

Key words: Polymerase Chain Reaction (PCR), *Salmonella*, Desiccated coconut (DC)

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Introduction

Sri Lanka is one of the pioneers in manufacturing the desiccated coconut and as per the reports it has commenced more than 107 years before using the technologies developed in Sri Lanka (S B Ratnayake, 2005). DC is the dried coconut kernel hygienically prepared in the form of flakes, threads and granules used as a food and manufactured mainly for export purposes. Sri Lanka plays a major role in the export of DC with the existing installed capacity of 100,000 MT per year (CDA statistics 2003). Desiccated coconut industry itself is a major contributor to the national economy of the Sri Lankan Government and in 2005 it's contribution is 19 million US\$.

Bacteria Salmonella is a major cause of food-borne infections in worldwide. Almost all vertebrates appear susceptible to the infection caused by the different species of *Salmonella* (Joseph *et al.*, 1999). Among 2000 known species, *S. typhi* and *S. paratyphi* are primarily human pathogens. Human infection is initiated by ingestion of a sufficient number of pathogen to overcome the defense mechanism in the human body system. Wilson *et al.* in 1955 reported that to cause a natural infection approximately 10^2 to 10^3 bacterium cells are sufficient. The extent of human infection is determined by the prevalence of bacterium in food and the availability of favorable conditions for the pathogen to further multiply in the contaminated food.

The *Salmonella* contamination of desiccated coconut causing an outbreak of typhoid fever was recorded for the first time in 1953 in Australia (Wilson *et al.*, 1955). Later, a survey carried out in the United Kingdom revealed that the 76 samples out of 851 samples of DC imported from Sri Lanka have been found contaminated with *Salmonella*, predominantly *S. paratyphi* (Galbraith 1960). The compulsory introduction of high stringent sanitary procedures into the Sri Lankan DC industry was a direct result of these findings and since then the incidence of contamination has diminished.

As a result, mandatory sanitary measures have been imposed to the DC industry in Sri

Lanka and each consignment of DC export accompanied a microbial quality certificate issued by a recognized authority indicating the absence of *Salmonella* by testing DC samples. The conventional culturing techniques and biochemical tests take an average 6 to 7 days to analyze a DC sample for *Salmonella* contamination. This delay in test procedure causes serious problems for the DC industry and some times urgent shipments made prior to obtaining the test results have been rejected by the importing countries. Thus there is a need to identify a rapid method to analyze DC for *Salmonella* contamination in order to minimize delays and avoid rejection of shipments.

Since the conventional bacteria cultural methodologies and biochemical tests for identification of bacterial species are time consuming and caused delays in export consignments, the necessity arises to look for a quick, accurate and fast detection technique. The polymerase chain reaction (PCR) is a biochemistry and molecular biology technique for isolating and exponentially amplifying a fragment of DNA, via enzymatic replication (Smithsonian Institution, 2006). PCR technique, a novel molecular biological method has been widely practiced to detect the microbial infections. This method has been successfully applied for the detection of various pathogens such as *Listeria monocytogenes*, *Camphylobacter* spp., *Vibrio* spp., *Escherichia coli*, *Shigella* spp. and *Salmonella* spp. in meat, fish, milk and processed food industries. PCR test results using *Salmonella* specific oligonucleotide DNA primers showed advance features, such as speed, specificity, sensitivity and accuracy in the detection of *Salmonella* spp. contaminated in food. The PCR is also easy to perform and can be used to analyze large numbers of samples compared to the biochemical methods (Stone *et al.*, 1994).

The purpose of this study is to establish a PCR-based detection technique as a quick, accurate and fast technique to detect *Salmonella* in DC samples to avoid delays in issuing microbial quality certificates.

Materials and method

The Desiccated Coconut samples were collected from the DC mills operated in the North Western Province in Sri Lanka as per the sampling methodology adopted by the Coconut Development Authority (CDA), Sri Lanka. The samples were drawn from the randomly selected 50kg DC bags on the basis of 10% from each batch of DC production packed in 50kg bags.

From each 50kg bag approximately 25g two separate samples were collected into sterilized polythene bags under the aseptic condition and then sealed by using the polythene sealer to avoid contaminations during the transportation to the laboratory.

Biochemical and serological methods of *Salmonella* detection and identification:

DC samples of more than 4000 were tested according to the conventional cultural methods consisting of pre-enrichment in Buffered Peptone Water (BPW), selective enrichment in Rappaport Vassiliadis Soya peptone (RVS) broth, plating on selective media; Xylose Lysine Decarboxylase (XLD) and Brilliant Green Bile Agar (BGA) for the detection of *Salmonella* bacteria. The suspected bacterial colonies grown on these selective medias were then tested by the api 20 E strips (Bio Meieux) and slide agglutination test with polyvalent 'O' and 'H' *Salmonella* antigen for conformity.

DNA extraction from five known bacterial cultures for PCR:

Methodology described by Guo *et al.*, (1998) was adopted in extraction of DNA from bacteria. Six known bacteria namely, *Salmonella* M1 type, *S. typhi*, *S. paratyphi*, *S. typhimurium*, *Bacillus subtilis* and *Escherichia coli* were cultured separately in nutrient agar plates and then the inoculated plates were incubated at 37°C for 24 hours. Single colonies grown in each plate were separately dissolved in 50µl of sterilized distilled water (SDW) contained in micro-

centrifuge tubes and then those bacterial cell suspensions were placed in a boiling water bath for 10 minutes.

The cell lysates in each micro-centrifuge tube were centrifuged at 13000 rpm for 30 seconds and the supernatants from each tube were taken separately. Supernatants were stored at -20°C to be used as the template DNA for PCR.

Preparation of DC samples as the positive controls:

The DC samples of 25g which was free from salmonella were separately inoculated with 100µl of *Salmonella* M1 type inoculums prepared in SDW by dissolving single colonies separately. The inoculated samples were subjected to pre-enrichment for 18 hours at 37°C in 125ml of buffered peptone water (BPW) and the DNA were extracted according to thermal treatment method.

Sensitivity tests for the detection technique:

Salmonella bacteria free DC samples of 25g were separately inoculated with 100µl of *Salmonella* M1 type and then prepared dilutions series of 10⁻¹ to 10⁻⁷ in SDW. The bacterial cell counts of these dilutions were also made as per the 'pour plate method' by inoculating nutrient agar plates with 100µl of each dilution separately. These cell counts were used to determine the sensitivity of PCR for the detection of *Salmonella*

Pre-enrichment of *Salmonella* in DC samples for DNA extraction:

The biochemical tests and serological methods were used to identified the *Salmonella* positive DC samples The samples of 25g were subjected to the pre-enrichment for 18 hours at 37°C in 125ml of BPW. The DNA was extracted according to the thermal treatment method. (Winters *et al.*, 1998).

Thermal treatment method for the extraction of DNA from *Salmonella* in pre-enriched DC samples:

The method was adopted from Winters *et al.*, 1998. Thirty milliliters of each pre-enriched samples were centrifuged at 1000 rpm for 10 minutes. The supernatants 25ml of each sample were separately taken and centrifuged again at 6000 rpm for 15 minutes. The supernatants in each sample were removed and the pellets were separately dissolved in 1 ml of SDW in micro-centrifuge tubes and centrifuged at 13000 rpm for 10 minutes. The supernatant in each tube were discarded and the pellets were dissolved in 100µl 1% Triton X-100 in SDW and boiled in a boiling water bath for 10 minutes.

The cell lysates were centrifuged at 13000 rpm for 30 seconds and the supernatants from each tube were taken separately and stored at -20°C to be used as the template DNA for PCR.

PCR for the detection of *Salmonella*:

The method adopted from Guo *et al.*, 1999. *Salmonella* specific primers for the conserved sequences of genes *InvA* and *InvE* have been used for PCR. These primer sequences are 5'TGCCTACAAGCATGAAATGG3' and 5'AAACTGGACCACGGTGACAA3'. The reactions were carried out in 25µl reaction mixtures each contained 2.5µl 10XPCR buffer (50mM KCl, 10mM Tris-HCl and 0.1% Triton X-100), 1µM of each primer, 100µM each of four deoxyribonucleotides, 1.5mM MgCl₂, 1.25 units of *Taq* DNA polymerase (Promega) 2µl of extracted DNA as the template. The thermal cycling programme was set in a TECHNE Flexi-Gene thermal cycler. The samples were initially

denatured at 94°C for 2 minutes. Thirty five cycles of amplification were run for 45 seconds at 94°C, 45 seconds at 50°C, 45 seconds at 72°C with the final extension continuing for 7 minutes. Each amplified products of 20µl aliquots separately mixed with 5µl loading dye were subjected to electrophoresis through 2.0% agarose gel containing ethidium bromide 0.5µg/ml. The amplified DNA products were visualized by exposing the gel to 312nm UV rays on the UV Transilluminator (Uvitec 312nm / STX-35MS) and photographed on a Polaroid film with a Polaroid camera (Polaroid – Gelcam).

Results and discussion

The reliability and specificity of the PCR method for detection of *Salmonella* was tested using extracted DNA from five known *Salmonella* species as the templates at the first step of developing this method. All *Salmonella* DNA were amplified generating a characteristic 457 bp product but not DNA of *E. coli* and *B. subtilis* used as controls (Figure 1) indicating the reliability of the test procedure and the specificity of the primers for *InvA* and *InvE* genes for the PCR detection of *Salmonella* species. The results are similar to those of Guo *et al.* (1998) who studied 71 *Salmonella* isolates belonging to five different serotypes.

Also, the PCR reactions with boiling-lyses extracted DNA from inoculated DC samples with known *Salmonella* spp. had generated 457 bp products without any non-specific amplification from *E. coli* and *B. subtilis* inoculated samples.

1 2 3 4 5 6 M



Figure 1: Agarose gel electrophoresis of the PCR amplified DNA for bacterial genomic DNA extracted from six known bacterial cultures. Lane 1: *Salmonella typhi*; Lane 2: *S. paratyphi*; Lane 3: *Bacillus subtilis*; Lane 4: *Salmonella* M1 type; Lane 5: *S. typhimurium*; Lane 6: Escherichia.

1 2 3 4 5 6 7 8 9 M

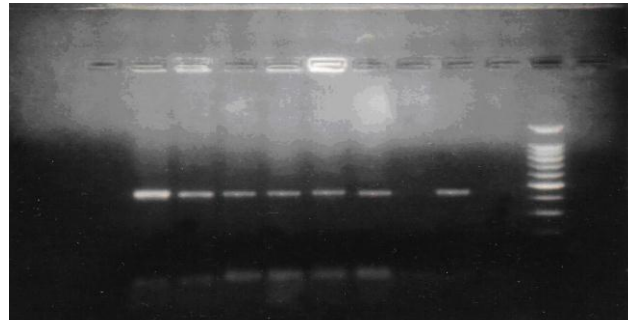


Figure 2: Agarose gel electrophoresis of the PCR amplified DNA for bacterial genomic DNA extracted from inoculated DC samples with serially diluted *Salmonella* M1 type. Lanes 1 to 7: The dilutions 10⁻¹ to 10⁻⁷; Lane 8: Positive control.

M 1 2 3 4 5

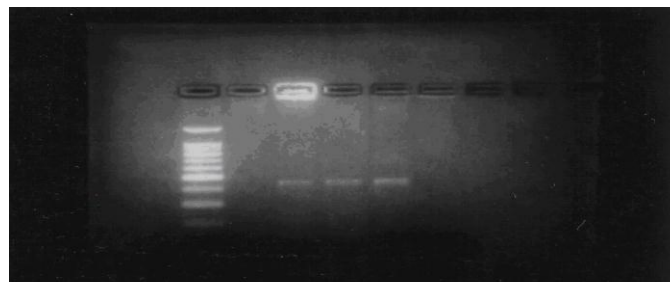


Figure 3: Agarose gel electrophoresis of the PCR amplified DNA for bacterial genomic DNA extracted from naturally contaminated DC samples. Lanes 2, 3 & 4: *Salmonella* positive; Lanes 1 & 5: *Salmonella* negative.

The DC samples containing known counts of *Salmonella* M1 type were used to determine the sensitivity of PCR for the detection of *Salmonella*. The bacterial counts of these samples were determined by 'pour-plate method' inoculating 100µl of each dilution into nutrient agar plates. Therefore, the PCR was carried out using the boiled lysates obtained from pre-enriched DC samples which were inoculated with known counts of *Salmonella* M1 type. The results revealed that the 10⁻¹ to 10⁻⁶ dilutions of the initial suspension of *Salmonella* cells inoculated

after 24 hours of pre-enrichment and boiling lysates DNA extraction method were positive for PCR detection of *Salmonella* M1 type (Figure 2) and produced the results within 24 hours. The conventional methods used at present showed similar detection limit of until 10⁻⁶ but produced the results only after 6-7 days. Estimation of viable bacterial count carried out using the pour-plate method revealed that 10⁻⁶ dilution of the inoculum consisted of 1000 CFU/ml. The 25g DC samples inoculated with 100µl volume of 10⁻⁶ dilution containing 100 cells. Therefore the analysis of DC

contaminated with *Salmonella* using the conventional and PCR methods are sensitive to detect as few as 4 CFU/g of DC contaminated with *Salmonella* M1 type.

The experiment carried out using naturally contaminated DC with *Salmonella* explain that the boiling-lyses extraction of DNA from the contaminated DC samples can be effectively done after employing the pre-enrichment and using 0.1% Triton X-100 to make the cell lysate. Resultant DNA bands from the PCR reactions with DNA extracted from three DC samples which have been confirmed positive with cultural and biochemical methods indicates that the developed PCR method is suitable for the routing detection of *Salmonella* in DC.

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