

EVALUATION OF FIVE DNA EXTRACTION METHODS IN THE DETECTION OF SALMONELLA ENTERICA FROM MEAT USING NESTED PCR

R.M.U.S.K.Rathnayaka¹

ABSTRACT

Polymerase Chain Reaction (PCR) based detection methods have received significant attention in food borne microbial pathogen detection. However, reliability and sensitivity of these methods are highly depending on the extraction of adequate amount of pure DNA using appropriate extraction method. Hence, selection of appropriate DNA extraction method is very important in PCR based detection of microbial pathogens. In this study, the extraction efficiency of five commonly used DNA extraction methods was evaluated. Salmonella enterica was used as experimental organism and five extraction methods were tested for their ability to extract DNA from spiked pork meat samples. Pork meat samples were incubated for four hours after being added a dilution series ($10^0 - 10^3$ CFU/mL) of Salmonella enterica culture. Then DNA was extracted from those samples by the five commonly used DNA extraction methods. Using extracted DNA, fliC gene of Salmonella was amplified by Nested PCR. Out of those five methods, the modified Fontana and Kapperd methods were found to be more effective in DNA extraction. In addition, those methods gave high detection sensitivity of 10^1 CFU/mL in Nested PCR amplification.

Keywords: DNA extraction; Nested PCR; Salmonella, Fontana, Kapperd

INTRODUCTION

Salmonella is a major concern as a pathogenic microorganism in food borne infections, causing mild to severe clinical effects (Jongerius-Gortemaker *et al.* 2002). They are Gram negative, motile, facultative anaerobic bacteria which typically causes an intestinal infection that is accompanied by fever, abdominal cramps and diarrhea. Contaminated egg, meat and poultry products are the main sources of *Salmonella* infections (Nowak *et al.* 2007; Wang *et al.* 1996). Contamination of sea foods by *Salmonella* is also a major public health concern (Shabarinath *et al.* 2007).

Inspection of food for the presence of *Salmonella* has become routine all over the world. Conventional microbiological methods used for the detection of *Salmonella* in foods generally require 72 to 96h (Tan & Shelef 1999). Such lengthy procedures are problematic for microbial detection in food industry, especially when applied for screening large numbers of food samples. A number of rapid methods for the detection of *Salmonella* in foods have been developed. This includes automated detection methods (Peng & Shelef 2001), immunological methods (Jouy *et al.*, 2005; Wang *et al.*, 1996) and nucleic acid based analyses (Whyte *et al.* 2002; Nam *et al.* 2005; Malorny *et al.* 2007). However, there are still problems

¹Department of Food Science and Technology, Faculty of Applied Science, Sabaragamuwa University of Sri Lanka, Belihuloya

with the sensitivity and specificity of those methods (Jenikova *et al.* 2000).

Polymerase chain reaction (PCR) based techniques are the most popular nucleic acid based techniques. These techniques are powerful diagnostic tools for the analysis of microbial infections as well as microorganisms present in food samples (Malorny *et al.* 2003). The PCR is a sensitive, rapid technique, in which a few copies of target DNA can be amplified to a level detectable by gel electrophoresis. The capacity of PCR to detect microorganisms depends on the purity of the template used as target and on the presence of a sufficient number of target molecules (Estrada *et al.* 2007).

The presence of PCR inhibitors in food samples and the minimum cell requirement are some of the limitations in PCR based assays leads to false negative results. The removal of inhibitory substances and rapid and efficient DNA extraction in the preparation of samples for PCR-based detection of food pathogens are important (Jenicova *et al.*, 2000). So, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction (Amagliani *et al.* 2007). The present study evaluates the efficiency of five different techniques used for DNA extraction from *Salmonella enterica* spiked in pork samples by their detection using nested PCR.

MATERIALS AND METHODS

Bacterial inoculum preparation

Salmonella enterica was cultured using Tryptic Soy Broth Yeast Extract medium (TSBYE) which contains 30g of tryptic soy broth powder with dextrose, 6g of yeast extracts and 1L of water. Cultures were incubated 4h at 36°C to mid-exponential growth phase and serially diluted (10^{-1} – 10^{-10}) in sterile distilled

water for enumeration. Bacteria were enumerated using Rambach agar plates at 37°C overnight and bacterial concentration was estimated by calculating the average number of red colonies on plates containing 30 to 300 colonies.

Bacterial dilutions contained 10^0 – 10^3 CFU/mL were prepared using the same mid-exponential phase bacterial culture. Five grams of pork sausage were inoculated by 1mL of each dilution, placed in 45mL of TSBYE medium and homogenized using a stomacher for 90s. Then the cultures were enriched for 4h at 37°C and used for DNA extraction followed by nested PCR.

DNA extraction

Enriched bacterial cultures were used for DNA extraction by five extraction methods. One milliliter of each culture was centrifuged at 12500rpm for 5min at 7°C. After centrifugation the pellet was obtained and used for DNA extraction by each extraction method.

The first extraction protocol was a method developed by Fontana *et al.* (2005) and modified by Estrada *et al.* (2007). The pellets obtained by centrifugation were washed with 200µL of ammonium hydroxide, 200µL of absolute ethanol, 400µL of petrol ether and 20µL of SDS (10%). The sample was then centrifuged at 12500rpm for 10 min at 4°C, and the pellet was re-suspended in a solution containing 200µL of 6M urea, 200µL of absolute ethanol, 400µL of petrol ether, 80µL of SDS (10%) and 13µL of 3M sodium acetate. A second centrifugation for 10 min at 12500rpm at 4°C was also performed, and the pellet was re-suspended with 600µL of TE buffer (Tris - EDTA) pH 8.0, 35µL of SDS (10%) and 10µL of DNase-free RNase (10 mg/mL). The tubes were incubated at 37°C for 30 min before the addition of 10µL of Proteinase K. This preparation was incubated at 37°C for 30 min. Finally,

130µL of 6M sodium perchlorate and 500µL of phenol chloroform isoamyl alcohol (25:24:1; pH 6.7) were added for DNA extraction. The tubes were then centrifuged at 12500rpm for 5 min, the aqueous phase was collected and the nucleic acids were precipitated with absolute alcohol. DNA was dissolved in 20µL of TE buffer.

The second DNA extraction protocol was a method developed by Kapperud *et al.* (1993) and modified by Estrada *et al.* (2007). The pellets obtained by centrifugation were re-suspended in 50µL of 1X PCR buffer containing 0.2mg of Proteinase K/mL. After being incubated at 37°C for 1h, the suspension was boiled for 10 min and then centrifuged at 12500rpm for 5 min at 4°C. The supernatant was used for performing the PCR.

The third protocol was Triton X - 100 method. The pellets obtained by centrifugation were diluted 1:10 with 1% - 2% Triton X - 100 and vortex. The suspension was heated in boiling water for 10min and the tubes were cooled to room temperature. Equal volume of chloroform was added to suspension and mixed well. The suspension was centrifuged at 12500rpm, 10min at room temperature. To the upper phase equal volume of 100% isopropyl alcohol was added and mixed gently by inverting the tube and then centrifuged for 10min at 12500rpm, 7°C. The DNA pellet obtained was washed with 1mL of 70% ethanol, centrifuged at 12500rpm, 5min, 7°C and freeze dried. Then dissolve in 20µL of TE buffer and store at -20 °C until use.

The fourth extraction method was a method proposed by Bansal *et al.* (1996). The pellets obtained by centrifugation were washed once with 1mL of pH 7.4 phosphate buffered saline (PBS), and re-suspended in equal volume of cold water and incubated in a boiling water bath for 10min. The clear supernatants obtained

after a 5 min of centrifugation at 12500rpm were stored at -20°C and used for PCR.

The fifth extraction method was the protocol proposed by Sambrook & Russel, (2001). The pellets obtained by centrifugation were re-suspended in 0.5mL of lysis solution (8M urea, 0.3M NaCl, 10mM Tris-HCl) with the addition of 0.5ml 10% SDS. After 20 min at 37 °C, DNA was extracted with 2 volumes of phenol by mixing in 10min and centrifuging at 12500rpm for 10min. An equal volume of chloroform:isoamyl alcohol was added to the aqueous phase and then samples were mixed as above and centrifuged at 12500 rpm for 5min.

The nucleic acids in the aqueous phase were then precipitated with 2.5 volumes of ethanol and 1/10 volume of 3M sodium acetate (pH 5.2) by incubating at -20°C for 1h and centrifugation. DNA pellets were washed with 70% ethanol and dissolved in 20µL of TE buffer and store at -20°C until use.

Determination of DNA quality and quantity

The quality of the extracted DNA by all methods was investigated by measuring absorbance at 260nm and 280 nm using spectrophotometer and calculating the A260 /A280 ratios with three replicates. DNA yield of extracted DNA was also measured using spectrophotometric readings with three replicates assuming that 1 OD at 260 nm equals 50 µg /ml DNA (Sambrook *et al.* 1989). Bacterial culture which contained 10³ CFU/mL was used for above experiment.

Nested PCR

The external and internal primers located on the conserved regions of the *fliC* gene which identified by Touron *et al.* (2005) was used for nested PCR. Those primers are;

5'-CGGCGTGAAAGTCCTGGCG-3'
 (Sal345),
 5'-GGTGTTAAAGTCCTGTCT-
 3'(Sal345d),
 5'-CGAATCTTCGATACGGCTACG-3'
 (Sal1312),
 5'CATCTTCGATACGGCTACG-3'
 (Sal1312d),
 5'-ATCCAGGTTGGTGCTAACG-3'
 (Salnes3d),
 5'-TTTACGGTTTGCCCAGG-3'
 (Salnes1),
 5'-CCGTATTGCCAAGGTTGG-3'
 (Salnes1d).

1µL of DNA extracted by different extraction methods were used for PCR amplification in a 50µL final volume of the following mixture: 25µL of PerfectShot™ Ex Taq (Takara), 0.25µM of each external primer (Sal345, Sal345d, Sal1312, Sal1312d), and sterilized distilled water. PCR amplification was performed with 10min at 95°C for denaturation, followed by a 35-cycle program (denaturation at 95°C for 1min, annealing at 55°C for 1min, extension at 72°C for 1min), and a final extension step at 72°C for 10min.

The nested PCR was processed using a 1µL volume of the previous PCR products following the same reaction mixture but with internal primer concentrations of 0.25µM of each internal primer: Salnes1, Salnes1d; and 0.5µM of the internal primer Salnes3d. Nested PCR amplification was performed with the same PCR protocol. The amplified DNA products were analyzed by gel electrophoresis in 2.5% agarose gel containing ethidium bromide in TAE buffer (20mM Tris acetate, 0.5mM EDTA,

pH 7.8). PCR products were visualized by UV illumination and their images were recorded.

RESULTS AND DISCUSSION

Detection of *Salmonella* in meat foods have involved culturing techniques (Hara-Kudo *et al.*, 2001), immunological test (Jouy *et al.*, 2005; Wang *et al.*, 1996) and nucleic acid based assays (Whyte *et al.*, 2002; Nam *et al.*, 2005; Malorny *et al.*, 2007). Comparing to those methods, PCR-based methods been recognized to be more sensitive, specific and rapid for food pathogen detection.

The quantity and purity of extracted nucleic acid are important factors in PCR-based detections. Selection of a proper method to extract appropriate amount of DNA containing minimum level of proteins, RNA or any other PCR inhibitors determines the success and the validity of the PCR analysis (Amaglini *et al.*, 2007). Other important factors which should be taken in to account when selecting a DNA extraction method are, the time need to complete the extraction and the toxicity and expenses of the chemical product employed in extraction (Chapela *et al.*, 2007).

Quality and quantity of extracted DNA

Purity of the extracted DNA was in the acceptable range (1.6 – 1.8) for all extraction methods (Table 1). Highest DNA yield was obtained by the modified Fontana *et al.* (2005) method while lowest yield from the Bansal *et al.* (1996) extraction method.

Table 01: Quantity and purity of extracted DNA by different extraction methods.

Extraction method	DNA yield (μg)	DNA purity (A_{260}/A_{280})
Modified Fontana et al. (2005) method	17.8 \pm 0.30	1.64 \pm 0.02
Modified Kapperud et al. (1993) method	15.6 \pm 0.10	1.64 \pm 0.01
Triton X - 100 method	14.8 \pm 0.46	1.77 \pm 0.03
Bansal et al. (1996) method	08.9 \pm 0.26	1.45 \pm 0.02
Sambrook & Russel, (2001) method	11.2 \pm 0.20	1.64 \pm 0.01

Nested PCR

DNA was extracted by five extraction methods from 4h enriched cultures obtained by initial inoculation of 10^3 to 10^0 in TSBYE medium. The extracted DNA was then amplified using the Nested PCR.

The detection of the amplified products of *fliC* gene by external primers required a minimum of 10^3 CFU/mL for all extraction methods tested. The second step PCR with internal primers for *fliC* gene increased the sensitivity of all methods except the method proposed by Sambrook & Russel, (2001).

The detection sensitivity of the modified Fontana *et al.* (2005) method and modified Kapperd *et al.* (1993) method were increased by 100 fold while increasing the detection sensitivity by 10 fold for other two methods (Figure 1).The modified

Fontana *et al.* (2005), and Kapperd *et al.* (1993) extraction methods tested in this experiment are found to be the best methods which could used in routine detection of *Salmonella enterica* from fork meat samples. Compared to other methods, the Bansal *et al.* (1996) method is simple, requires less harmful chemicals and is less time consuming. Nested PCR of DNA extracted by this method could be applied for preliminary testing of samples with high possibility to be positive for *Salmonella*.

The enrichment step which was reduced to 4h in this experiment compared to overnight enrichment practice to obtain detectable number of cells this could decrease the overall experimental time to one working day compared to two working days of reported nested PCR applications (Amaglini *et al.*, 2007) and 7-10 days of standard conventional microbiology methods.

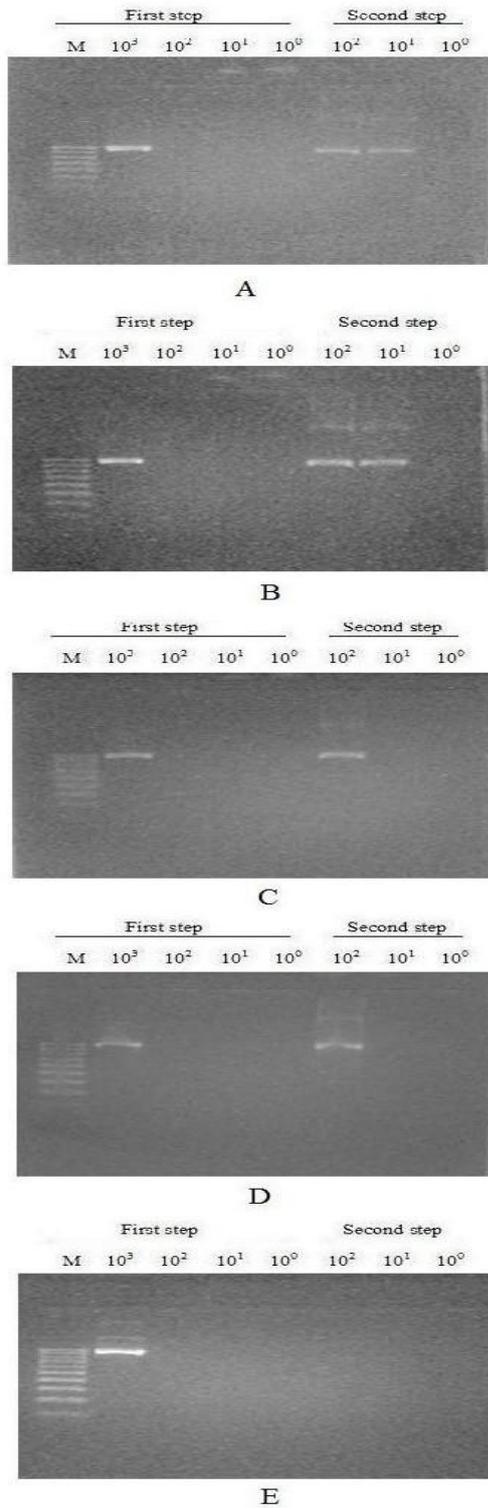


Figure 01. Electrophoresis agarose gel stained with ethidium bromide of Nested PCR products (First step 948bp and second step 892bp) for *fliC* gene for the DNA extracted by five selected extraction methods. M, 100bp molecular weight marker, 10³ to 10⁰, bacterial concentrations (CFU/mL). A, B, C, D and E are different DNA extraction methods, Fontana *et al.* (2005), Kapperud *et al.* (1993), Triton X – 100, Bansal *et al.* (1996) and Sambrook & Russel, (2001) respectively.

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