SIMULTANEOUS DETECTION OF FOUR FOOD BORNE BACTERIAL PATHOGENS BY METAL HYDROXIDE IMMOBILIZED MULTIPLEX PCR METHOD

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ABSTRACT

***Salmonella enterica, Vibrio parahaemolyticus, Escherichia coli and Listeria monocytogenes* are human pathogenic bacteria which could be contaminated with many types of food, especially meat products. Testing of food for those pathogens has become routine practice all over the world. However, since the available traditional detection methods are time consuming and labor intensive, there is a need for rapid, sensitive, specific and cost effective detection technique. In this study, a rapid and sensitive single tube Multiplex PCR method for simultaneous detection of those four bacterial pathogens by amplification of target genes *gyrB* of *V. parahaemolyticus*, *invA* of *S. enterica*, *eaeA* of *E.coli* and *hly* of *L. monocytogene* was established. Concentration of bacterial cells from food samples for DNA extraction is another problem in testing of food pathogens by PCR techniques. Metal hydroxide immobilization was applied prior to DNA extraction and found to be effective step to overcome this problem.**

INTRODUCTION

As consequence of many pre-harvest, harvest and post harvest factors, food and water can be contaminated by human pathogenic microorganisms. *Salmonella enteritica, Vibrio parahaemolyticus, Escherichia coli and Listeria monocytogenes* are four food and water borne pathogenic bacteria causing gastroenteritis of human. *Salmonella* is one of the major food and water borne pathogenic bacteria which cause the disease called salmonellosis. Contaminated egg, meat and poultry products are the main sources of infection by *Salmonella* (Wang *et al.* 1996; Nowak *et al.* 2007). *Vibrio parahaemolyticus* is a gram negative bacterium considered as a major cause of gastroenteritis specially associated with the consumption of raw seafood (Pham *et al.* 2007). *E. coli* is a bacterium present in the intestinal tract of warm blooded animals as its normal micro flora. Most of the *E. coli* strains are non-pathogenic but some serogroups, such as entero-hemorrhagic O157:H7, are pathogenic and cause severe diarrhea and fever (Stender *et al.* 2001). *E. coli* can colonize the intestinal tract of cattle and other animals. During slaughtering, they may contaminate the carcass and subsequently be distributed via cut meat or raw meat materials intended for further processing. *L. monocytogenes* is an opportunistic intracellular pathogen causes the disease called listeriosis. It is considered as an important cause of human food and water borne infections worldwide (Liu 2006).

Conventional methods of the detection of bacterial pathogens in foods generally based on the identification of bacteria using selective culture media by their morphological, biochemical and immunological characteristics (Wang *et al.* 2007). These methods are tedious, time consuming, and possible to have errors in sampling and enumeration when low number of microorganisms present in the sample. Moreover, it is not possible to use these methods in detection of viable but non-culturable microorganisms (Kong *et al.* 2002). As a replacement for these conventional methods there are number of rapid methods developed, including automated detection methods (Peng *et al.* 2001), immunological methods (Wang *et al.* 1996; Jouy *et al.* 2005) and nucleic acid based analyses (Whyte *et al.* 2002; Nam *et al.* 2005; Malorny *et al.* 2007). However, nucleic acid based assays are more popular at present.

Out of all nucleic acid based detection methods, polymerase chain reaction (PCR) based methods have been identified as a powerful diagnostic tool for the detection of pathogenic microorganisms (Malorny *et al.* 2003). Compared to other detection methods, these methods are rapid, highly specific and sensitive in the identification of target organisms (Wang *et al.* 2007). Recently, multiplex PCR which is a modified PCR method was introduced and widely applied in microbial detection which can be used to detect more than one organism by one PCR reaction simultaneously. The establishment of Multiplex PCR protocol is a tedious and time consuming since it needs lengthy optimization

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procedure, but after being established it is rapid and low cost method for microbial pathogen detection. The multiplex PCR method has been used by many scientists to detect different types of pathogenic microorganisms in food and water (Pham *et al.* 2007; Petra *et al.* 2007).

PCR based detection mainly depends on the purity and amount of the template DNA used (Estrada *et al.* 2007). The presence of PCR inhibitors in food samples and incomplete bacterial cell isolation lead to the production of false negative results in PCR based detection and the removal of PCR inhibitors, efficient bacterial cell isolation and efficient DNA extraction is important (Jenicova *et al.* 2000). Therefore, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction (Amagliani *et al.* 2007) and efficient isolation of bacterial cells from food samples by immobilization.

In our experiment, a multiplex PCR method was developed for the detection of four bacterial pathogens, *S. enterica, V. parahaemolyticus, .E. coli and L. monocytogenes*. The detection sensitivity of those pathogens from inoculated raw meat samples was also studied. Metal hydroxide immobilization prior to DNA extraction was used to bacterial cell concentration from food samples.

METHODOLOGY

Bacterial cultures
V. parahaemolyticus, S. enterica, E. coli, and *L. monocytogenes* were cultured for 6h at 36^0C using universal culture medium, Tryptic Soy Broth Yeast Extract medium (TSBYE). The cultures were then serially diluted (10^{-1}–10^{-10}) in sterile distilled water and enumerated in Tryptic Soy Agar (TSA) plates. Bacterial concentration was estimated by calculating the average number of colonies on plates containing 30 to 300 colonies.

DNA extraction to establish Multiplex PCR method
Dilutions of each of the four bacteria which contained 10^7cfu/mL were prepared and 1mL of each dilution was used to inoculate 25g of raw pork meat sample. Another 25g of raw pork meat sample was inoculated with 1mL of each dilution. Then each inoculated sample was placed in 225mL of TSBYE medium and homogenized using a stomacher in 90s. Those cultures were incubated for 6h at 36^0C. After incubation, bacterial DNA was extracted from each culture following the method developed by Fontana *et al.* (2005) and modified by Estrada *et al.* 2007. Bacterial cell pellets were obtained by centrifugation of 1mL of each culture at 12,000rpm for 10min at 4^0C. The resulting pellets were first

dissolved in 200µL of Ammonium hydroxide followed by 200µL of absolute ethanol, 400µL of petrolium ether and 20µL of 10% SDS. Then they were centrifuged at 12,000rpm for 10min at 4^0C again, and the resulted pellets were re-suspended in a solution containing 200µL of 6M urea, 200µL of absolute ethanol, 400µL of petrolium ether, 80µL of 10% SDS and 13µL of 3M Sodium acetate. Another centrifugation for 10min at 12,000rpm at 4^0C was also performed, and the pellets were re-suspended with 600 µL of pH 8.0 TE buffer (Tris - EDTA), 35µL of 10% SDS and 10 µL of 10 mg/mL DNase-free RNase. The tubes were incubated at 37^0C for 30min before adding 10µl of Proteinase K and then again incubated at 37^0C for 30min. 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 12,000rpm for 5min, the aqueous phase was collected and the nucleic acids were precipitated with absolute alcohol. The extracted DNA was dissolved in 20µL of TE buffer.

DNA extraction for the study of the sensitivity of Multiplex PCR method and the effect of metal hydroxide immobilization
Ten mixed bacterial dilution of four bacteria which contained 10^0 to 10^{10}cfu/mL of each bacterium were prepared and used to inoculate ten 25g raw-pork-meat samples. Then were placed in 225mL of TSBYE medium and homogenized using a stomacher in 90s and were incubated for 6h at 36^0C. The incubated samples were used to extract DNA by the method developed by Fontana *et al.* (2005) and modified by Estrada *et al.* (2007) as mentioned above with and without metal hydroxide immobilization prior to DNA extraction. In this study, the immobilization method proposed by Lucore *et al.* (2000) was used with some modifications. Hundred micro liters of bacteria was added to 200µL of Zirconium hydroxide and agitated at room temperature. Then it was vortex and centrifuged at 2000 rpm for 5min at 7^0C. After centrifugation the pellet was obtained and used for DNA extraction.

Integrity and purity of extracted DNA
Five micro liter of each extracted DNA was visualized by gel electrophoresis in a 0.9% agarose gel stained with ethidium bromide (0.5µg/mL). After electrophoresis at 80V for 40min DNA bands were visualized by using ultraviolet light and documented by a gel documentation system. The quality of the extracted nucleic acids was also investigated, and the absorbencies at 260 and 280nm were measured using spectrophotometer and the A_{260} /A_{280} ratios were calculated for all DNA samples.

PCR amplification

Extracted DNA were amplified by PCR using four specific forward and reverse primers, CGGCGTG GGTGTTTCGGTAGT and TCCGCTTCGCGCT CATCAATA for *gyrB* gene of *V. parahaemolyticus* (Venkateswaran *et al.* 1998), TCTCTACTTAAC AGTGCTGC and TGGTATAAGTAGACAGGG GC for *invA* gene of *S. enterica* (Hoa *et al.* 2004)*,* GACCCGGCACAAGCATA AGC and CCACC TGCAGCAACAAGAGG for the *eaeA* gene of *E. coli* (Paton *et al.* 1998)*,* and TATACCACGGA-GATGCAGTG and GCCGAAGTTTACATTCAA GC for *hly* gene of *L. monocytogene* (Hoa *et al.* 2004). 2.5μL of each extracted DNA was used as a template for PCR amplification in a 50μL final volume of the following mixture: 25μL of Perfect-Shot^{TM} Ex Taq (from Takara), 0.25μM of each primer and sterilized distilled water. PCR reactions were performed with the initial denaturation at 95^{0}C for 3min followed by 35 cycles of denaturation at 95^{0}C for 30s, annealing at 55^{0}C for 60s, and extension at 72^{0}C for 1.5min, and final extension for 7min at 72^{0}C.

Gel electrophoresis and gel documentation

All PCR products were visualized by gel electrophoresis. Ethidium bromide containing 2.5% agarose gel in TAE buffer (20mM Tris acetate, 0.5mM EDTA, pH 7.8) was used. The gel electrophoresis processes were carried out for 2.5h at the constant voltage condition of 35mV. After gel electrophoresis, the PCR products were visualized by UV illumination using gel documentation system and images were recorded.

RESULTS AND DISCUSSION

The extraction of the adequate amount of the pure total DNA is a basic requirement in PCR based detection. In this study, the gel electrophoresis of the

Figure 1: PCR amplification of four bacterial pathogens DNA. M: 100bp molecular size marker, 1: *Vibrio parahaemolyticus* (685bp), 2: *Escherichia coli* (482bp), 3: *Listeria monocytogene* (384bp), 4: *Salmonella enterica* (285bp), 5: DNA mixture of all four pathogens.

extracted total DNA showed clear single bands which indicate the good integrity of the extracted DNA. The purity of the extracted DNA was also in the acceptable range. The ratios of the absorbance at 260nm to the absorbance at 280nm were in the range of 1.6 to 1.8 for all extracted DNA samples. The specificity of primers used to detect target gene is very important in the detection of pathogenic organisms from a food sample which could contain a mixture of microorganisms, and this is a major concern in establishment of Multiplex PCR protocols. The primers used in this study were tested for their specificities. The sensitivity of the detection depends on the condition of the PCR reaction such as, primer annealing temperature, primer concentration, Mg^{2+} concentration, extension time and characteristics of DNA polymerase used. In this study, PerfectShot^{TM} Ex Taq (from Takara) master-mix was used with the optimum conditions based on the reported applications of the same primers. The used primers were able to amplify successfully the target genes of the extracted DNA from meat samples which were inoculated with 10^{7}cfu/mL bacteria cultures and enriched for 6 h in TSBYE medium, so that no non-specific band was observed in gel electrophoresis. The bacterial DNA extracted from meat samples inoculated with the four bacteria separately were able to produce bands of 285bp, 685bp, 384bp and 482bp for the gene gyrB of Vibrio parahaemolyticus, invA of *S. enterica*, eaeA of *E.coli* and hly of *L. monocytogene* respectively, while the bacterial DNA extracted from meat samples inoculated with a mixture of all of the four bacteria produced the all above four bands (Fig. 1). This results indicated the suitability of the above four primers in a Multiplex PCR for the simultanious detection of four pathogens, *V. parahaemolyticus*, *S. enterica*, *E. coli* and *L. monocytogene*.

Detection sensitivity is another important factor concerned in the evaluation of microbial pathogen detection methods. This experiment assessed the ability of metal hydroxide immobilization in increasing the detection sensitivity by increasing the

Figure 2: PCR amplification of four pathogenic bacterial DNA extracted without metal hydroxide immobilization. M: 100bp molecular size marker, from 1 to 10: bacterial concentrations from 10^{10} to 10^{0}cfu/mL respectively

Figure 3: PCR amplification of four pathogenic bacterial DNA extracted with metal hydroxide immobilization. M: 100bp molecular size marker, from 1 to 10: bacterial concentrations from 10^{10} to 10^0 cfu/mL respectively

concentration of cells from food sample. The developed Multiplex PCR method was able to detect all of the four microorganisms up to 10^4 cfu/mL for the DNA extracted without metal hydroxide immobilization (Fig. 2). But when metal hydroxide immobilization was applied, the detection sensitivity was increased by 10 fold, 10^3 cfu/mL (Fig.3).

Although the infectious dose varies with different pathogen types, it is generally believed that most bacterial pathogens are able to cause infection when more than 10^3 infectious cells are ingested. So, the detection sensitivity of the developed Multiplex PCR method with metal hydroxide immobilization of cells prior to DNA extraction is within the infectious dose of most enteric pathogens (Kong *et al.* 2002). However, this developed Multiplex PCR method should be improved further to achieve higher sensitivity when concerning high risk pathogens.

CONCLUSIONS

The Multiplex PCR method developed in this study could provide a powerful supplement to conventional methods for more accurate, rapid and sensitive risk assessment and the monitoring of selected four pathogenic bacteria associated with meat and meat products. The metal hydroxide immobilization could be used as an effective application in increasing the concentration of bacterial cells from food samples in order to increase the detection sensitivity. This Multiplex PCR method should be further improved for the detection of other important food and water borne pathogenic bacteria, and the effectiveness of metal hydroxide immobilization should be tested for different food matrix.

REFERENCES

Amagliani G, Giammarini C, Omiccioli E, Brandi G and Magnani M 2007 Detection of Listeria monocytogenes using a commercial PCR kit and different DNA extraction methods. Food Control. 18, 1137 –1142.

Estrada C, Lucero SM, Carmen LD, Genaro SD and Guzman AMSD 2007 Comparison of DNA extraction methods for pathogenic Yersinia enterocolitica detection from meat food by nested PCR. Food Research International, 40, 637 –642.

Fontana C, Cocconcelli PS and Vignolo G 2005 Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *International Journal of Food Microbiology, 103,* 131 –142.

Hoa NTH 2004 Multiplex polymerase chain reaction (PCR) for the detection of food pathogens. Master thesis, Asian Institute of Technology, Thailand.

Jeníkova G, Pazlarova J and Demnerova K 2000 Detection of *Salmonella* in food samples by the combination of immunomagnetic separation and PCR assay. Internatl Microbiol, 3:225–229.

Jouy E, Proux K, Humbert F, Rose V, Lalande F, Houdayer C, Picault JP and Salvat G 2005 Evaluation of a French ELISA for the detection of Salmonella Enteritidis and Salmonella Typhimurium in flocks of laying and breeding hens. Preventive Veterinary Medicine. 71, 91–103.

Kong RYC, Lee SKY, Law TWF, Law SHW and Wu RSS 2002 Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. Water Research, 36, 2802-2812.

Lucore LA, Cullison MA and Jaykus EA 2000 Immobilization Metal Hydroxide as a mean to concentrate food born bacteria for detection by cultural and molecular methods. Applied and Environmental Microbiology, 1769 -1776.

Liu D 2006 Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. Journal of Medical Microbiology, 55, 645–659.

Malorny B, Bunge C and Helmuth R 2007 A real-time PCR for the detection of Salmonella Enteritidis in poultry meat and consumption eggs. Journal of Microbiological Methods 70, 2, 245-251.

Malorny B, Tassios PT, Radstrom P, Cook N, Wagner M and Hoorfar J 2003 Standardization of diagnostic PCR for the detection of foodborne pathogens. International Journal of Food Microbiology 83, 1, 25, 39-48.

Nam H, Srinivasan V, Gillespie BE, Murinda SE and Oliver SP 2005 Application of SYBR green real-time PCR assay for specific detection of Salmonella spp. In dairy farm environmental samples. International Journal of Food Microbiology. 102, 161– 171.

Nowak B, Muffling TV, Chaunchom S and Hartung J 2007 Salmonella contamination in pigs at slaughter and on the farm: A field study using an antibody ELISA test and a PCR technique. International Journal of Food Microbiology 115 259 – 267.

Paton AW and Paton JC 1998 Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic *E. coli* hlyA, rfb0111, and rfb0157. Journal of Clinical Microbiology, 36, 598-602.

Peng H and Shelef LA 2001 Automated simultaneous detection of low levels of listeriae and salmonellae in foods. International Journal of Food Microbiology, 63, 225–233.

Petra FG, Wolffs Glencross K, Norling B, Mansel and Griffiths W 2007 Simultaneous quantification of pathogenic Campylobacter and Salmonella in chicken rinse fluid by a flotation and real-time multiplex PCR procedure. International Journal of Food Microbiology. 117, 50 – 54.

Pham HN, Ohkusu K, Miyasaka J, Sun XS and Ezaki T 2007 Rapid and specific identification of 5 human pathogenic Vibrio species by multiplex polymerase chain reaction targeted to dnaJ gene. Diagnostic Microbiology and Infectious Disease. *59, 3, 271-275*.

Stender H, Oliveira K, Rigby S, Bargoot F and Coull J 2001 Rapid detection, identification, and enumeration of *Escherichia coli* by fluorescence in situ hybridization using an array scanner. Journal of Microbiological Methods, 45, 31–39.

Venkateswaran K, Dohmoto N and Harayama S 1998 Cloning and nucleotide sequence of the gyrB gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimo. Applied and Environmental Microbiology, 681-687.

Wang H, Blais BW, Brooks BW and Yamazaki H 1996 *Salmonella* detection by the polymyxin-cloth enzyme immunoassay using polyclonal and monoclonal detector antibodies. International Journal of Food Microbiology, 29, 31-40.

Wang L, Li Y and Mustapha A 2007 Rapid and simultaneous quantification of *Escherichia coli* O157:H7, *Salmonella,* and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. Journal of Food Protection, 70, 6, 1366-1372.

Whyte P, Gill KM, Collins JD and GormLey E 2002 The prevalence and PCR detection of Salmonella contamination in raw poultry. Veterinary Microbiology, 89, 53–60.