*Journal of Food and Drug Analysis, Vol. 16, No. 4, 2008, Pages 37-43* 藥物食品分析 第十六卷 第四期

# Development of a Multiplex PCR Method for the Detection of Six Common Foodborne Pathogens

**IUN-FAN LEI[1]*, PAUL ROFFEY[2], CHRIS BLANCHARD[2] AND KEN GU[1]**

*[1] School of Health Sciences, Macao Polytechnic Institute, Macao SAR, People's Republic of China*
*[2] Charles Sturt University, Wagga Wagga NSW 2678, Australia*

(Received: June 29, 2007; Accepted: December 21, 2007)

## ABSTRACT

This study developed a multiplex PCR method for the screening and detection of six common foodborne pathogens in Macao. The m-PCR procedure, which uses six pairs of primers, produced specific amplicons of the expected sizes from mixed populations of reference bacterial strains in food samples and from pure cultures. The verocytotoxin (*stx*) gene of *Escherichia coli* O157: H7, the hemolysin (*hly*) gene of *Listeria monocytogenes*, the invasion (*invA*) gene of *Salmonella* spp., the cholera toxin (*ctx*) gene of *Vibrio cholerae*, the thermolabile hemolysin (*tlh*) gene of *V. parahaemolyticus*, and the thermostable nuclease (*nuc*) gene of *Staphylococcus aureus* were used as target genes for m-PCR detection. The detection limit of the assay for the bacterial targets was 1-100 cfu per mL. The m-PCR analysis was designed for three main food clusters; meat and meat products testing for *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157: H7, seafood and seafood products testing for *V. cholerae* and *V. parahaemolyticus* and ready-to-eat foods testing for *S. aureus*. Overall, results of the present study indicate that the m-PCR is a potential technique for the rapid detection of foodborne bacteria for routine monitoring and risk assessment of food.

Key words: foodborne, bacterial pathogens, multiplex PCR

## INTRODUCTION

Surveillance of foodborne diseases is of an increasingly high priority in the public health agenda worldwide. Bacterial contamination of food represents one of the major public health problems. *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Vibrio cholerae*, and *V. parahaemolyticus* are the main pathogens involved in food poisoning and are routinely monitored in Macao[(1)]. Some of these foodborne pathogens can cause life-threatening diseases to humans and animals. Whilst all are well recognized, some are considered emerging because they have recently become more common.

*Salmonella* spp., *S. aureus*, *E. coli* O157: H7 and *L. monocytogenes* are the predominant bacteria species that cause public health problems worldwide. In addition, *V. cholerae* is a common bacterium in raw or under processed seafood which can cause very severe diseases, and has been endemic in Asia and Africa for years. *V. parahaemolyticus* is also a frequent cause of food poisoning in seafood, thus *V. cholerae* and *V. parahaemolyticus* are significant pathogens that require testing in seafood or related products.

The Food & Drug Administration's Bacteriological Analytical Manual (BAM)/AOAC international standard culture methods[(2)] are used in the government laboratories of Macao as the golden standard. A number of immunological protocols have also been developed for the detection of bacterial pathogens. The VIDAS enzyme-linked immunofluorescent assay (ELFA) is the official AOAC method for the screening of *Salmonella* spp., *E. coli* O157: H7 and *L. monocytogenes* in routine samples, but the tests are mono-specific hence separate tests for each bacterium. Since commercial kits are unavailable for *V. cholerae* and *V. parahaemolyticus*, traditional culture-based methods are required, which are time-consuming, tedious, low throughput and invariably mono-specific (detecting only one type of pathogen at a time). It takes at least four days to identify the species and more than four media for enumeration and selective culture for each pathogen.

The use of molecular methods has provided highly sensitive detection methods for specific pathogens in environmental samples. Numerous studies have been published on m-PCR detection of foodborne pathogens including pathogenic *E. coli*[(3)], *E. coli* O157: H7[(4)], *Salmonella* and *Shigella*[(5)], *V. parahaemolyticus*[(6)], *E. coli*, *S.* Typhimurium and *Vibrio* spp.[(7)], *S. aureus* and *Yersinia enterolitica*s[(8)], *Listeria* spp.[(9)] *Campylobacter jejuni* and *Arcobacter butzleri*[(10)].

Most of these m-PCR studies have been developed for foodborne pathogens commonly encountered in the reported areas or artificially inoculated[(11)]. Hence, it is necessary to develop m-PCR protocols which fit the local

* Author for correspondence. Tel: +853-66837838; 853-3998626
Fax: +853-28753159; E-mail: iflei@ipm.edu.mo

situation. In this study an m-PCR assay was designed to detect the six most common foodborne pathogens in Macao. The m-PCR test detects *Salmonella* spp., *L monocytogenes*, and *E. coli* O157: H7 in meat and meat products, *V. cholerae* and *V. parahaemolyticus* in seafood and seafood products, and *S. aureus* in ready-to-eat foods.

## MATERIALS AND METHODS

### I. *Bacterial Strains and Cultural Methods*

The bacterial strains examined in this study are listed in Table 1. Sources were obtained from the American Type Culture Collection, World Health Organization, local hospitals and municipal laboratory. All bacterial strains were cultured in tryptic soy broth yeast extract broth (TSBYE) at 35°C[12]. The TSBYE contained 30 g of tryptic soy broth powder (Difco Laboratories, Detroit, MI, USA), 6 g of yeast extract, and 1 litre of water.

### II. *Food Sample Preparation*

Food samples were either purchased from local food stores, collected by Health Bureau, or obtained from local food microbiology laboratory and homogenized follow-

**Table 1.** Bacterial strains for the evaluation of specificity of PCR primers in this study

| | Strain | Source | *ctxAB* | *hly* | *invA* | *nuc* | *stx* | *tlh* |
|---|---|---|---|---|---|---|---|---|
| 1 | *Aeromonas sobria* | Lab strain[a] | – | – | – | – | – | – |
| 2 | *E. coli* | ATCC 25922 | – | – | – | – | – | – |
| 3 | *E. coli* | ATCC 35218 | – | – | – | – | – | – |
| 4 | *E. coli* O157:- | Lab strain[a] | – | – | – | – | – | – |
| 5 | *E. coli* O157:H7 | UK strain | – | – | – | – | + | – |
| 6 | *E. coli* O157:H7 | WHO strain | – | – | – | – | + | – |
| 7 | *E. coli* HB101 | BioRad kit | – | – | – | – | – | – |
| 8 | *Vibrio alginolyticus* | Lab strain[a] | – | – | – | – | – | – |
| 9 | *Vibrio cholerae* non O1 | Lab strain[a] | – | – | – | – | – | – |
| 10 | *Vibrio cholerae* O1 | Lab strain[b] | + | – | – | – | – | – |
| 11 | *Vibrio cholerae* O139 | Lab strain[b] | + | – | – | – | – | – |
| 12 | *Vibrio fluvialis* | Lab strain[a] | – | – | – | – | – | – |
| 13 | *Vibrio parahaemolyticus* | ATCC 17802 | – | – | – | – | – | + |
| 14 | *Vibrio vulnificus* | Lab strain[b] | – | – | – | – | – | – |
| 15 | *Enterobacter cloacae* | ATCC 23355 | – | – | – | – | – | – |
| 16 | *Klebsiella pneumoniae* | ATCC 13883 | – | – | – | – | – | – |
| 17 | *Proteus mirabilus* | Lab strain[a] | – | – | – | – | – | – |
| 18 | *Pseudomonas aeruginosa* | ATCC 27853 | – | – | – | – | – | – |
| 19 | *Salmonella* Typhi | Lab strain[a] | – | – | + | – | – | – |
| 20 | *Salmonella* Typhimurium | ATCC 14028 | – | – | + | – | – | – |
| 21 | *Serratia marcescens* | ATCC 8100 | – | – | – | – | – | – |
| 22 | *Shigella flexneri* | ATCC 25929 | – | – | – | – | – | – |
| 23 | *Enterococcus faecalis* | ATCC 29212 | – | – | – | – | – | – |
| 24 | *Listeria innocua* | Lab strain[a] | – | – | – | – | – | – |
| 25 | *Listeria monocytogenes* | ATCC 13932 | – | + | – | – | – | – |
| 26 | *Staphylococcus aureus* | ATCC 25923 | – | – | – | + | – | – |
| 27 | *Staphylococcus epidermidis* | ATCC 12228 | – | – | – | – | – | – |
| 28 | *Streptococcus pyogenes* | ATCC 19615 | – | – | – | – | – | – |

[a] Isolated from environmental specimens.
[b] Isolated from clinical specimens.

ing the standard methods[2,13]. 90 mL of TSBYE broth was added to 10 g of food. Food samples were homogenized in a blender and incubated overnight at 35°C.

III. *DNA Extraction*

Aliquots (1.0 mL) of various cultures or food sample were centrifuged at 9000 x g for 3 min in a Hermle 2160M centrifuge. The pellet was then resuspended in 500 µL of TE buffer (pH 7.8) and vortexed. Various food clusters focused on different bacterial groups, hence each food cluster had a different pretreatment method for DNA extraction. For meat or meat product samples, an extraction suitable for *L. monocytogenes* was used. This involved a pre-incubation with 5 µL of lysozyme (Sigma Cat. # L7651, 10 mg per mL) at 37°C for 40 min, followed by 5 µL of proteinase K (Sigma, 15 mg per mL) at 55°C for 30 min[13]. For ready-to-eat food samples, lysostaphin was added to lyse *S. aureus* cell walls. The food sample pellet was resuspended in 500 µL of TE with 3 µL of lysostaphin (Sigma Cat. # L7386, 3.46 U per µL), incubated for 30 min at 37°C, then 30 µL of 0.1% SDS added and incubated for additional 30 min at 37°C[13]. For seafood or seafood product samples, no specific pre-treatment procedure was performed. All the samples were finally lysed for 10 min in a boiling water bath, then the lysate was cooled in an ice bath. Aliquot (2 µL) of the extract was used as the template for PCR amplification.

IV. *PCR Primers*

Oligonucleotide primers, ranging from 20- to 24-mers, were selected from either the FDA's BAM standard methods or previously reported studies[2,14-18,21]. All oligonucleotide primers used in the present study were synthesized by Invitrogen Life Technologies Limited (Japan) and were of desalted grade. The six PCR primer sets used in this study for m-PCR, their corresponding gene targets and size of expected amplification products are shown in Table 2.

V. *Uniplex and Multiplex PCR Amplification*

Uniplex and multiplex amplifications were made in a 50-µL reaction mixture, which contained 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs deoxynucleoside triphosphate, 2.5 U of *Taq* Polymerase (Takara Premix Taq™ Version, Dalian, China), and 0.25 mM of each specific primer. 2 µL of template was added to the reaction mix.

All PCR amplification reactions were performed in a DNA thermal cycler (Applied Biosystem 9700, Califonia, USA). An initial incubation at 95°C for 3 minutes was used to denature the template and activate the *Taq* polymerase. Then 35 cycles of the following PCR temperature cycling parameters was performed: denaturation for 60 s at 95°C, primer annealing for 90 s at 55°C, and DNA extension for 90 s at 72°C. Following amplification, a final extension of the incompletely synthesized DNA was carried on at 72°C for 10 min, then the reaction cooled and maintained at 4°C. Negative control reaction mixtures contained sterile distilled water in place of template DNA.

PCR products were analyzed by gel electrophoresis in 1.7% agarose containing 0.5 mg/mL ethidium bromide in TBE buffer (89 mM Tris-HCl pH 8.3, 89 mM boric acid, 2.5 mM EDTA). The DNA bands were observed by irradiating the pre-stained gel under UV illuminator at 302 nm and photographed.

This study was monitored by the use of reaction controls. The Quality Control Positive (QCP) contained

**Table 2.** Primer sequences and expected size of PCR-amplified gene targets of six species of foodborne pathogens

| Species | Target Gene | Primer - Nucleotide sequence (5' to 3') | PCR product Size (bp) | Reference |
|---|---|---|---|---|
| *Vibrio cholerae* | *ctxAB* | TGA AAT AAA GCA GTC AGG TG<br>GGT ATT CTG CAC ACA AAT CAG | 777 | (17) |
| *E. coli* O157:H7 | *stx* | TGG GTT TTT CTT CGG TAT CC<br>CCA GTT CAG AGT GAG GTC CA | 632 | (21)<br>(modified from ref) |
| *Salmonella* spp. | *invA* | TAC TTA ACA GTG CTC GTT TAC<br>ATA AAC TTC ATC GCA CCG TCA | 570 | (16)<br>(modified from ref) |
| *Vibrio* parahaemolyticus | *tlh* | CGG ATT ATG CAG AAG CAC TG<br>ACT TTC TAG CAT TTT CTC TGC | 444 | (17)<br>(modified from ref) |
| *Staphylococcus aureus* | *nuc* | GCG ATT GAT GGT GAT ACG GTT<br>AGC CAA GCC TTG ACG AAC TAA AGC | 270 | (14) |
| *Listeria monocytogenes* | *hly* | GCA TCT GCA TTC AAT AAA GA<br>TGT CAC TGC ATC TCC GTG GT | 174 | (15) |

a sufficient amount of each pathogen to give a positive result across all primer sets (approx $10^4$-$10^5$ cfu per species); this was processed through the entire protocol. All target pathogens were used for the QCP. The Quality Control Negative (QCN) contained a sufficient amount of closely related, but non-target strains and was also processed through the entire protocol. *Citrobacter freundii*, *E. coli*, *L. innocua*, *S. epidermidis*, and *V. cholerae* non-O1 were chosen as QCNs for *Salmonella* spp., *E. coli* O157: H7, *L. monocytogenes*, *S. aureus*, and *V. cholerae* and *V. parahaemolyticus* respectively. Contamination was monitored through a Quality Control Blank (QCB), which contained sterile distilled water in place of template DNA in the PCR reaction mix[19].

VI. *Food Sampling Evaluation*

In order to verify the validity of the m-PCR method for detection of pathogens in real food samples, food samples were spiked with reference bacteria at known concentrations and subject to extraction and m-PCR analysis. All food samples were evaluated by using Vidas ELFA screening kit (BioMerieux, France) for *E. coli* O157: H7, *Salmonella* spp. and *L. monocytogenes* in meat or meat products, FDA's BAM method for *E. coli* O157: H7, *Salmonella* spp. and *L. monocytogenes* in meat or meat products, for *V. cholerae* and *V. parahaemolyticus* in seafood and seafood products; and Petrifilm STX method for *S. aureus* in ready-to-eat foods as the standard method to demonstrate the outcome of m-PCR. Template DNA was prepared as described previously.

To evaluate sensitivity of the m-PCR method, known bacterial counts ($10^0$-$10^4$ cells) of each pathogen were added to 1 mL of food sample homogenates immediately prior to DNA extraction.

## RESULTS AND DISCUSSION

I. *Identification and Primer Design*

The multiplex PCR primer set targeted at the *invA*, *hly*, *stx*, *nuc*, *ctx*, and *tlh* genes were chosen for the simultaneous detection of *Salmonella* spp., *L. monocytogenes*, *E. coli* O157: H7, *S. aureus*, *V. cholerae*, and *V. parahaemolyticus* respectively[2,14-18,21]. Amongst the six sets of PCR primers, the *V. parahaemolyticus* primers were the only primer set that was not targeted at a virulence-associated gene. Table 2 lists the primers used for the amplification of these genes and the expected sizes of the amplicons.

The design and theoretical analysis of primers with respect to self-complementarity, inter-primer annealing and optimum annealing temperatures were accomplished by using the online software Primer 3[22] and IDT SciTools Oligoanalyzer 3.0[23]. Computer predictions indicated that all oligonucleotide primer pairs were specific for their target genes. To facilitate PCR product detection, the primers were designed such that the expected sizes of the amplification products of each target gene would be different (Table 2) to permit size discrimination by gel electrophoresis (Figure 1).

II. *Determination of Specificity of the Multiplex PCR Protocol*

In order to evaluate and verify the specificity of the primers in this study, each primer pair was tested by PCR on DNA templates prepared from a panel of 28 different bacterial isolates (including the quality control strains). The analysis indicated that all primer pairs were specific for their corresponding target organisms (Table 1).

PCR amplification with each set of oligonucleotide primers yielded a single, detectable DNA fragment of the expected molecular weight only in the presence of each of their respective template DNAs (Figure 1), whereas other strains did not show any product. These results indicated that each of the selected oligonucleotide primer sets was specific for its respective target microbial pathogen, and that the DNA extraction method yielded sufficient DNA template to permitted m-PCR to detect the six target pathogens. This specificity provided an appropriate foundation upon which PCR-based detection of the pathogens could be developed, and in which ''false positive'' signals would be unexpected. The m-PCR was tested for its specificity against other bacteria such as *E. coli*, *Klebsiella* spp., *Aeromonas* spp. and *Enterobacter cloacae*. Only target species gave the characteristic bands.

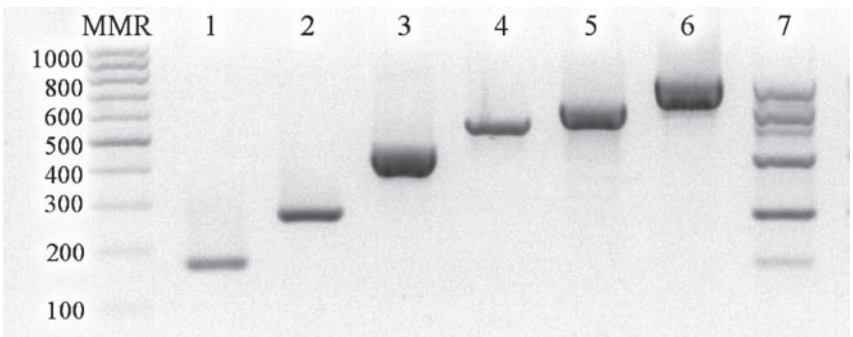


**Figure 1.** Electrophoretic analysis of amplified DNA from reference bacterial species obtained in individual and m-PCR protocols under optimal conditions. Lane MMR, 100-bp DNA ladder marker (Takara, Dalian, China); lane 1, *Listeria monocytogenes* (ATCC 13932); lane 2, *Staphylococcus aureus* (ATCC 25923); lane 3, *Vibrio parahaemolyticus* (ATCC 17802); lane 4, *Salmonella Typhimurium* (ATCC 14028); lane 5, *E. coli* O157: H7 (WHO strain); lane 6, *Vibrio cholerae* O1 (lab strain) and lane 7, m-PCR product of all targeted species.

*Journal of Food and Drug Analysis, Vol. 16, No. 4, 2008*

### III. *Determination of Detection Sensitivity of the Multiplex PCR Protocol*

To determine sensitivity of the m-PCR protocol, overnight-enumerated samples of all six foodborne pathogens were serially diluted 10 fold and cell lysates were used as templates for m-PCR amplification. Each pathogen was diluted from $10^4$, $10^3$, $10^2$, $10^1$ and $10^0$ bacteria. The results of PCR showed that the lowest amount of template was detected in one bacterial cell in *E. coli* O157: H7 and 10 to 100 for the others pathogens. Detection limit of *V. cholerae* and *V. parahaemolyticus* are shown in Figure 2. The level of detection was equivalent to 10 to 100 cells for each target pathogens.

### IV. *Multiplex PCR Analysis of Food Samples*

The method developed involves homogenization of food samples, extraction of DNA and simultaneous amplification of target genes. A total of 77 meat and meat products, 145 seafood and seafood products, and 56 ready-to-eat food samples were analyzed between February 2006 and March 2007. The number of samples detected in which pathogens is listed in Table 3.

For the 77 meat and meat related samples, we got ideal m-PCR results compared to Vidas ELFA assays and FDA's BAM cultural methods. All positive isolates including 2 *Salmonella* spp., 2 *E. coli* O157: H7 and 2 *L. monocytogenes* were detected after overnight enrich-

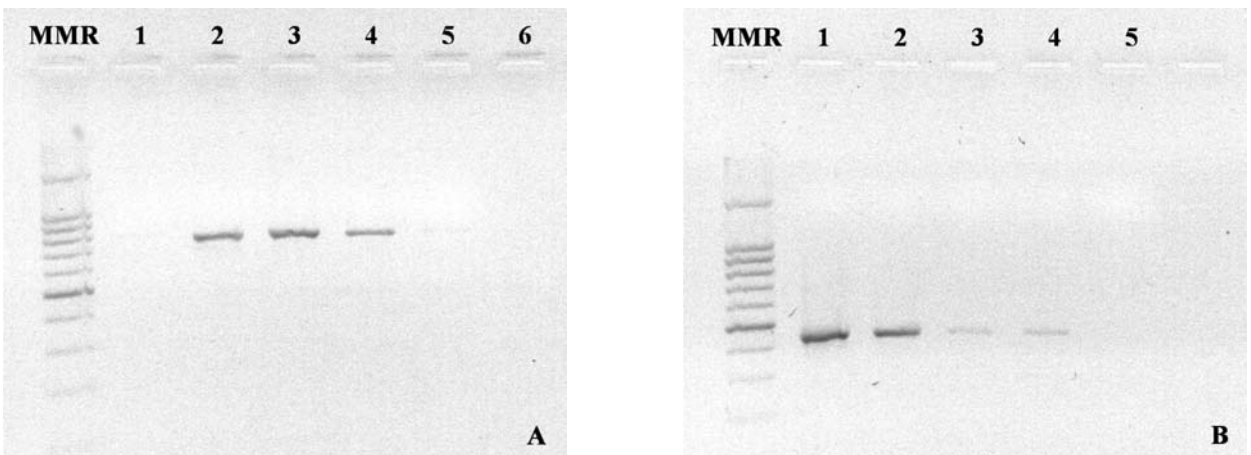


**Figure 2.** Agarose gel showing detection limit of multiplex PCR assay for detection of each foodborne pathogen in terms of number of bacterial cell. Lane MMR, 100-bp DNA ladder marker (Takara, Dalian China); In panel A: lane 1 is sterile water, lane 2 to 6 are *V. cholerae* O1 (lab strain) ($10^4$, $10^3$, $10^2$, $10^1$ and $10^0$ bacteria); panel B: lane 1 to 5 are *V. parahaemolyticus* ATCC 17802 ($10^4$, $10^3$, $10^2$, $10^1$ and $10^0$ bacteria) per mL of homogenate respectively.

**Table 3.** Comparison of m-PCR method and cultural methods for detection of six foodborne pathogens

| | No. of samples | No. of Positive samples by | | |
|---|---|---|---|---|
| | | m-PCR | ELFA | Culture |
| Meat and meat samples | 77 | | | |
| *Salmonella* spp. | | 9 | 4 | 2 |
| *Listeria monocytogenes* | | 3 | – | 2 |
| *E. coli* O157:H7 | | 3 | 6 | 2 |
| Seafood and seafood samples | 145 | | | |
| *Vibrio cholerae* | | 0 | – | 9[a] |
| *Vibrio parahaemolyticus* | | 103 | – | 30 |
| Ready-to-eat food samples | 56 | | | |
| *Staphylococcus aureus* | | 49 | – | 47 |

[a] All 9 isolates were *Vibrio cholerae* non-O1, non-O139.

ment in TSBYE broth. Both Vidas ECO and m-PCR methods could detect the two isolates of *E. coli* O157: H7 from cultural method. However, Vidas ECO was targeted on *E. coli* O157 only, and a total of 6 *E. coli* O157 were screened out. Considering *E. coli* O157: H7 was more virulent, and identify the *stx* gene implicated the predominant serotype in disease. Those 4 *E. coli* O157 not detected by m-PCR methods were H7 and *stx* gene negative strains. Using m-PCR method for detection *E. coli* O157: H7 is more specific for toxigenic strains. One *Salmonella* positive isolate (not shown in Table 3) in seafood samples was detected with our 6-primer set, and confirmed with cultural method.

A total of 9 *V. cholerae* isolates from seafood and seafood products were confirmed by Vitek GNI+ (BioMerieux, France). Following further identification by serological typing, all were confirmed as *V. cholerae* non-O1, non-O139 strains. According to FDA's BAM standard, *V. cholerae* isolated from foods or the environment do not produce cholera toxin (CT) and are not considered virulent. The failure to detect these cells in the seafood samples further demonstrated the desired specificity of the m-PCR protocol for the pathogenic *V. cholerae* 01 and 0139 strains. The relative high rate of positives for *V. parahaemolyticus* in seafood samples demonstrated a significant improvement in detection of *V. parahaemolyticus* over traditional culture procedures. However, it also indicated that the m-PCR procedure can be improved to include targets for the *V. parahaemolyticus* toxin genes, since tests for the presence of the toxin genes (*tdh* and *trh* genes) of *V. parahaemolyticus* using FDA's BAM method were all negative.

Like *V. parahaemolyticus*, the *S. aureus* target gene is species specific but not specific for pathogenic strains. As a consequence it was not surprising to have a very high positive rate in the ready-to-eat food products tested. This also demonstrated that the m-PCR could be refined with an additional test for the staphylococcal enterotoxin gene.

Various studies have been published on m-PCR detection of foodborne pathogens, including species differentiation, regional specific, and artificially inoculated studies[11]. In conclusion, based on 278 food samples, our results demonstrate that our m-PCR method compares favorably with the commercial kits and standard culture methods. In addition, our m-PCR procedure can be employed as a routine procedure for the screening of *Salmonella*, *E. coli* O157: H7, *L. monocytogenes*, *V. cholerae*, *V. parahaemolyticus* and *S. aureus* in a diverse range of food types. The high throughput and cost-effective m-PCR protocol developed in this study could provide a powerful supplement to conventional methods. Its ability to rapidly monitor various types of microbial pathogens would be extremely useful not only for routine assessment of food hygiene to protect public health, but also for the rapid assessment of suspected food poisoning cases.

The major contribution of this study is the development of a protocol for the simultaneous screening and detection of several important bacterial species which are a public safety concern in foods.

## ACKNOWLEDGEMENTS

This study was supported by the funds provided by Macao Sciences and Technology Development Fund (No. 032/2005/A), the Macao SAR Government.

We thank Health Bureau, Municipal Laboratory, and Macao Polytechnic Institute for kindly providing food samples, bacterial strains and laboratory facilities.

## REFERENCES

1. Laboratório de Saúde Pública. 2006. Microbiologia - Nº. de Análises Realizadas por Tipo de Amostras e Parâmetros. In " 2006 Boletim Estatístico", Serviços de Saúde, Macau SAR; Annu. Rep: 89
2. USFDA/CFSAN. 1998. FDA Bacteriological analytical manual In. 8ath ed. AOAC International. Gaithersburg, MD
3. Bottero, M. T., Dalmasso, A., Soglia, D., Rosati, S., Decastelli, L. and Civera, T. 2004. Development of a multiplex PCR assay for the identification of pathogenic genes of *Escherichia coli* in milk and milk products. Mol. Cell Probes. 18: 283-288.
4. Fratamico, P. M., Sackitey, S. K., Wiedmann, M. and Deng, M. Y. 1995. Detection of *Escherichia coli* O157: H7 by multiplex PCR. J. Clin. Microbiol. 33: 2188-2191.
5. Vantarakis, A., Komninou, G., Venieri, D. and Papapetropoulou, M. 2000. Development of a multiplex PCR detection of *Salmonella* spp. and *Shigella* spp. in mussels. Lett. Appl. Microbiol. 31: 105-109.
6. Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C., Jones, D. D. and Kaysner, C. A. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J. Microbiol. Methods. 36: 215-225.
7. Brasher, C. W., DePaola, A., Jones, D. D. and Bej, A. K. 1998. Detection of microbial pathogens in shellfish with multiplex PCR. Curr. Microbiol. 37: 101-107.
8. Ramesh, A., Padmapriya, B. P., Chrashekar, A. and Varadaraj, M. C. 2002. Application of a convenient DNA extraction method and multiplex PCR for the direct detection of *Staphylococcus aureus* and *Yersinia enterocolitica* in milk samples. Mol. Cell Probes. 16: 307-314.
9. Bansal, N. S., McDonell, F. H., Smith, A., Arnold, G. and Ibrahim, G. F. 1996. Multiplex PCR assay for the routine detection of *Listeria* in food. Int. J. Food. Microbiol. 33: 293-300.
10. Winters, D. K. and Slavik, M. F. 2000. Multiplex PCR detection of *Campylobacter jejuni* and *Arcobacter butzleri* in food products. Mol. Cell Probes. 14: 95-99.

11. Kim, J. S., Lee, G. G., Park, J. S., Jung, Y. H., Kwak, H. S., Kim, S. B., Nam, Y. S. and Kwon, S. T. 2007. A novel multiplex PCR assay for rapid and simultaneous detection of five pathogenic bacteria: *Escherichia coli* O157: H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*. J. Food Protect. 70: 1656-1662.
12. Wang, R. F., Cao, W. W. and Cerniglia, C. E. 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. 83: 727-736.
13. Farber, J. M., Gendel, S. M., Tyler, K. D., Boerlin, P., Landry, W. L., Fritschel, S. J. and Barrett, T. J. 2001. Molecular Typing and Differentiation. In "Compendium of methods for the Microiological examination of foods". 4th ed. pp. 331-342. Downes, F. P. and Ito, K. eds. American Public Health Association. Washington, D.C.
14. Brakstad, O. G., Aasbakk, K. and Maeland, J. A. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol. 30: 1654-1660.
15. Deneer, H. G. and Boychuk, I. 1991. Species-specific detection of *Listeria monocytogenes* by DNA amplification. Appl. Environ. Microbiol. 57: 606-609.
16. Jung, W. S., Kim, S., Hong, S. I., Min, N. K., Lee, C. W. and Paek, S. H. 2004. DNA probe chip system for multiple detection of food poisoning microorganisms. Mater. Sci. Eng. 24: 47-51.
17. Koch, W. H., Payne, W. L. and Cebula, T. A. 1995. Detection of enterotoxigenic *Vibrio cholerae* in foods by the polymerase chain reaction. In "FDA Bacteriological Analytical Manual". 8th ed. pp. 28.01-28.09. AOAC International. Gaithersburg, MD.
18. Ziebell, K. 2003. MFLP-86 Identification of presumptive positive verocytotoxigenic *Escherichia coli* by the polymerase chain reaction. In "Food Directorate's - Laboratory procedure" Health Canada. Canada.
19. Malorny, B., Tassios, P. T., Radstrom, P., Cook, N., Wagner, M. and Hoorfar, J. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 83: 39-48.
20. Kong, R. Y., Lee, S. K., Law, T. W., Law, S. H. and Wu, R. S. 2002. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. Water Res. 36: 2802-2812.
21. Lin, Z., Kurazono, H., Yamasaki, S. and Takeda, Y. 1993. Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. Microbiol. and Immunol. 37: 543-548.
22. Rozen, S. and Skaletsky, H. J. 2000 Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386.
23. Integrated DNA Technologies SciTools Oligoanalyzer 3.0 [Internet] Coralville, IA: Available from: http://www.idtdna.com/SCITOOLS/scitools.aspx