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Multiplex PCR for Detection of *Staphylococcus aureus* and *Listeria monocytogenes* in Ready-to-eat Foods

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Abstract

Foodborne diseases are common problems worldwide. *Staphylococcus aureus* is one of the most important food-borne pathogens. *Listeria monocytogenes* is widely found in contaminated foods, especially in refrigerated and ready-to-eat foods. Rapid detection and identification are needed to prevent and control the food contamination by these infectious microorganisms. For the objective of the research, multiplex PCR technique has been developed for rapid detection of *S. aureus* and *L. monocytogenes* in ready-to-eat foods. In this study, two-pair of primers were designed within conserved regions of the virulence genes, *coa* gene of *S. aureus* and *prfA* gene of *L. monocytogenes* and then were used for detection of those bacteria. The results showed that this multiplex PCR could detect at least 1 ng of *S. aureus* DNA and 150 pg of *L. monocytogenes* DNA. Investigation into the artificially contaminated foods, this multiplex PCR was able to detect less than 10^4 cells/g of *S. aureus* and 1 cell/g of *L. monocytogenes* in foods. In addition, there were no amplifications of nucleic acids from other food related-pathogens, indicating the specificity of this test. Detections in thirty ready-to-eat food samples from local markets in Chonburi province, Thailand, showed that none of them were contaminated with *S. aureus* and *L. monocytogenes*. Therefore, this finding indicated good hygiene in production of ready-to-eat foods in these areas. Consequently, this multiplex PCR can be further developed and employed for monitoring of *S. aureus* and *L. monocytogenes* in contaminated foods.

Introduction

Foodborne disease is defined by the World Health Organization as a disease of infectious or toxic nature caused by the consumption of contaminated food or water. It is still a major concern worldwide (Ananchaipattana et al., 2012; Laaksonen et al., 2017; Mercado et al., 2012; Paudyal et al., 2017). Staphylococcal food poisoning is one of the most common food-borne diseases resulting from the ingestion of enterotoxigenic strains of *Staphylococcus aureus*. *S. aureus* is a facultative anaerobic gram-positive coccus found in the air, dust, sewage, water and environmental surfaces. *S. aureus* belongs to the normal flora found on the skin and mucous

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membranes of mammals and birds. Some strains of *S. aureus* are able to produce enterotoxin which is the causative agent of staphylococcal food poisoning. The foodstuff or ingredients can be contaminated with toxins in various temperatures that allow the growth of *S. aureus.* However, staphylococcal enterotoxins are highly heat resistant. Many foods can be a good medium growth for *S. aureus* including milk, butter, ham, sausages, cheese, meat, salad, and cook meals. Pasteurization can kill *S. aureus* but has no effect on enterotoxin. The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting followed by diarrhea. *Listeria monocytogenes* is a gram positive facultative intracellular bacterium found in soil, silage, groundwater sewage and vegetation. *L. monocytogenes* has become an important food-borne pathogen distributed in dairy products, undercooked meat, poultry, seafood, vegetables, ice cream, and especially refrigerated and ready-to-eat foods (Chen et al., 2016; Wilson, 1995). Various ready-to-eat foods contaminated with *L. monocytogenes* have been linked to outbreaks and illnesses (Garner & Kathariou, 2016). In a healthy person, *L. monocytogenes* causes gastroenteritis which can resolve itself without treatment. Whereas immunocompromised individuals, the bacterium can cause systemic infections that lead to meningitis, encephalitis (Barocci et al., 2015). *L. monocytogenes* synthesizes a number of secreted virulence factors whose expression is regulated by the transcriptional activator *prfA*. Moreover, it has become a serious issue to the food industry due to its ability to survive in most common food processing conditions such as high pH, high salt concentration, low water content, and refrigeration temperature. The microbiological food testing is necessary to prevent food contamination and outbreaks of foodborne illness. Commonly food products are usually contaminated at low levels (i.e. <100 CFU/g). However, up to now there is still not a specific method for testing foods.

Conventional microbiological methods for detection and identification of the pathogens in foods involve culture methods regarding enrichment and biochemical tests, which are usually very sensitive. However, these methods need a long time such as 3 to 5 days. Molecular methods based on multiplex polymerase chain reaction (PCR) technology for rapid detection of these pathogens have been developed (D'Amico & Donnelly, 2008, 2009; Kumar et al., 2009). These methods can reduce the time for detection of contaminated foods. In this study, multiplex PCR based on using primers for *coa* gene which encode coagulase, a major virulent factor of *S. aureus* and *prfA* gene encoding a transcriptional activator and is required for expression of virulence gene of *L. monocytogenes,* were developed and used for detection of those bacteria in ready-to-eat foods. Therefore, the aim of this research is to develop the multiplex PCR for detecting foodborne pathogens, *L. monocytogenes* and *S. aureus.* Several local ready-to-eat foods were tested for contamination by using this method.

Materials and methods

1. Microorganisms

S. aureus, L. monocytogenes, Escherichia coli and *Vibrio cholerae* used in this study were obtained from Faculty of Allied Health Sciences, Burapha University. Bacteria were grown in LB medium at 37°C for 18 h.

2. Primer design

The primers targeting of *coa* and *prfA* gene were designed by Bioedit (version 7.0.4) based on the National Center for Biotechnology Information (NCBI). Primers were analyzed by Oligos (version 9.1) and Standard Nucleotide BLAST for the specification. The forward and reverse primers; F-5'GGGATAACAAAG CAGATGCG-3', R-5'-ACGTTGATTCAGTACCTT GTG-3', amplify a 1,353 bp fragment of *coa* gene of *S. aureus.* The forward and reverse primers; F-5'GAG TATTAGCGAGAACGGGA-3', R-5'-TAACAGCT GAGCTATGTGCG-3', amplify a 420 bp fragment of *prfA* gene of *L. monocytogenes.*

3. DNA extraction

Bacterial DNAs were extracted by commercial DNA extraction kit (GF-1 Bacterial DNA Extraction Kit) and tested for the sensitivity of monoplex PCR and multiplex PCR.

4. Monoplex PCR

Each primer pair was used as a specific primer for detection of each bacterial specie. The primer pair for *coa* gene was used to amplify *S. aureus* DNA. The primer pair for *prfA* gene was used to amplify *L. monocytogenes* DNA. The PCR reaction consisting of *Taq* buffer with KCl, 0.2 mM of dNTPs, 1 µM of primer for *coa* gene or primer for *prfA* gene, 0.5 U of *Taq* DNA polymerase (DyNAzyme II DNA polymerase, Finnzymes Oy, Finland) and 1 µl of DNA template, in a final volume of 25 µl. Negative control of amplification was performed with nuclease-free water instead of the DNA template. Each PCR reaction were done in a thermal cycler

(GeneAmp PCR System 9700 (PE Applied Biosystems, Norwalk, CT, USA). Amplification condition including initial denaturation at 94 °C for 2 min and 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min. A final extension was performed for 5 min at 72 °C.

5. Multiplex PCR

The multiplex PCR was carried out using specific two-pair of primers. The forward and reverse primers; F-5'-GGGATAACAAAGCAGATGCG-3', R-5'- AC GTTGATTCAGTACCTTGTG-3', amplify a 1,353 bp fragment of *coa* gene of *S. aureus*. The forward and reverse primers; F-5'-GAGTATTAGCGAGAACGG-GA-3', R-5'-TAACAGCTGAGCTATGTGCG-3', amplify a 420 bp fragment of *prfA* gene of *L. monocytogenes*. PCR amplification was conducted in a solution containing *Taq* buffer with KCl, 0.2 mM of dNTPs, 1.2 µM of each primer for *coa* gene and 1 µM of each primer for *prfA* gene, 0.5 U of *Taq* DNA polymerase (DyNAzyme II DNA polymerase, Finnzymes Oy, Finland) and 1 µl of DNA template, in a final volume of 25 µl. Negative control of amplification was performed with nuclease-free water instead of the DNA template. Reactions were carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, Norwalk, CT, USA) thermal cycler. Amplification following initial denaturation at 94 °C for 2 min was performed in 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. A final extension was performed for 5 min at 72 °C.

6. Artificially contaminated food samples

Sensitivity assays were carried out for artificially inoculated sample. *S. aureus* and *L. monocytogenes* were grown in LB medium at 37°C for 18 h, after cell counting by haemacytometer, the cells were 10-fold diluted in LB medium ranging from 1 to 10^6 cells/ml. Cell counting was confirmed by colony plate count. One milliliter of each dilution or LB medium only as negative control was added into 5 grams of ready-to-eat food (sushi) in 13 ml of LB medium. The foods were sterilized by autoclaving before being artificially contaminated. The samples were homogenized and enriched by incubation at 37°C for 6 h. One milliliter of the enriched sample was taken and centrifuged at 500 rpm for 5 min to eliminate food particles and then at 6000 rpm for 5 min to collect bacterial cells, finally the pellets were washed twice with 0.8% NaCl and subjected to DNA extraction with Qiagen DNA extraction kit.

7. Naturally contaminated food samples

Thirty ready-to-eat foods including sushi, sausage, undercooked meat, dairy products, refrigerated and ready-to-eat foods were randomly purchased from local markets in Chonburi province during January to February 2015 and freshly processed. Before the investigation of bacterial contamination by the multiplex PCR, samples were homogenized, enriched and prepared by the process as described above.

8. DNA analysis by gel electrophoresis

Amplified products were analyzed by electrophoresis through 1% agarose gel. One microliter of the PCR product was stained with SYBR gold and analyzed by gel electrophoresis in 1% agarose gel at 110 V for 25 min. GeneRuler 100 bp (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA marker.

Results

1. Detection of *S. aureus* and *L. monocytogenes* by specific primers

The primer pair synthesized for *S. aureus* allowed PCR amplification of a 1,353 bp. Figure 1 shows the result obtained when *S. aureus* DNA was amplified and tested in the PCR using specific primer for *coa* gene of *S. aureus*. Amplification of *L. monocytogenes* DNA using the designed primer pair results in presence of 420 bp product. Therefore, both *S. aureus* and *L. monocytogenes* strains can be amplified while negative control were not amplified (Fig. 1). Moreover, other bacteria such as *E. coli* and *Vibrio* sp. did not presence the amplification (data not shown).

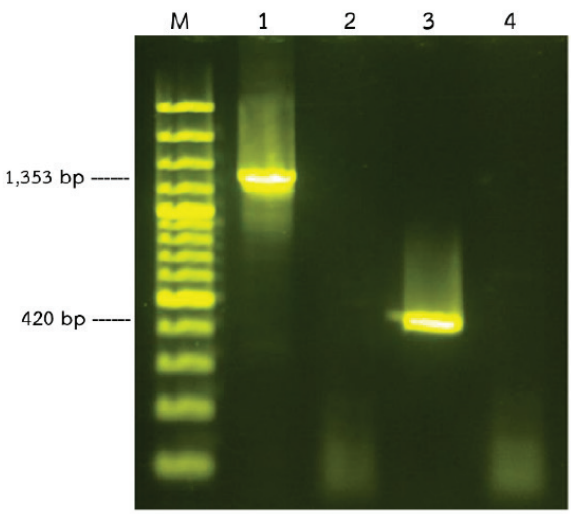


Fig. 1 Amplified products from purified *S. aureus* and *L. monocytogenes* DNA by using its specific primer. (lane M: 100 bp DNA marker, lane 1: *S. aureus* DNA, lane 3: *L. monocytogenes* DNA, lane 2 and 4: negative control (use DNAase-free water instant of bacterial DNA)

2. Detection of *S. aureus* and *L. monocytogenes* by multiplex PCR

Specific primer pair for *coa* gene of *S. aureus* and *prfA* gene of *L. monocytogenes* were used for multiplex PCR. The conditions of the multiplex PCR were optimized as described in the method. When both purified *S. aureus* and *L. monocytogenes* DNA were used as template, it showed that PCR products of 1,353 bp and 420 bp were amplified from *S. aureus* and *L. monocytogenes,* respectively (Fig. 2, lane 1).

Specificity of the multiplex PCR was tested with other food pathogens including *E. coli* and *V. cholerae. S. aureus, L. monocytogenes* and *E. coli* or *V. cholerae.* They were performed with the multiplex PCR in the same condition. The result showed that there was no amplification from *E. coli* or *V. cholerae* (Fig. 2, lane 2 and 3). Therefore, these *coa* and *prfA* primer pairs have more specificity for *S. aureus* and *L. monocytogenes.*

The investigation of sensitivity was tested by different amounts of each DNA, the results showed that the multiplex PCR can detect at least 1 ng of *S. aureus* DNA (Fig. 3) and 150 pg of *L. monocytogenes* DNA (Fig. 4).

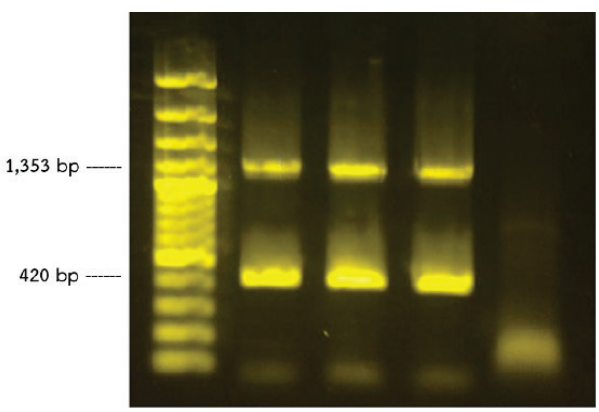


Fig. 2 Amplified products from *S. aureus* DNA (1,353 bp) and *L. monocytogenes* DNA (420 bp) by using the multiplex PCR. (lane M: 100 bp DNA marker, lane 1 : PCR products from *S. aureus* and *L. monocytogenes* DNA; lane 2: PCR products from *S. aureus, L. monocytogenes,* and *E. coli* DNA; lane 3: PCR products from *S. aureus*, *L. monocytogenes*, and *V. cholerae* DNA; lane 4: negative control)

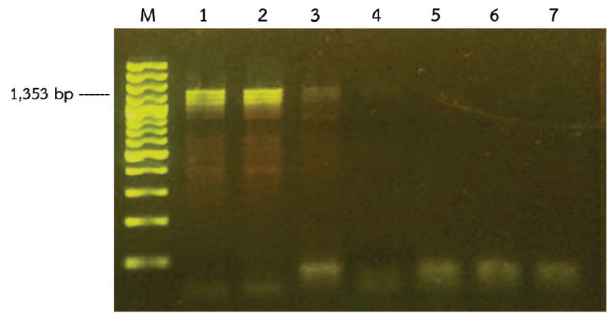


Fig. 3 Sensitivity of the multiplex PCR for detecting of *S. aureus* DNA. Different amounts of *S. aureus* DNA from 1 µg to 10 pg was used as template for the multiplex PCR. (lane M: 100 bp DNA marker, lane 1-7: 1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and negative control, respectively)

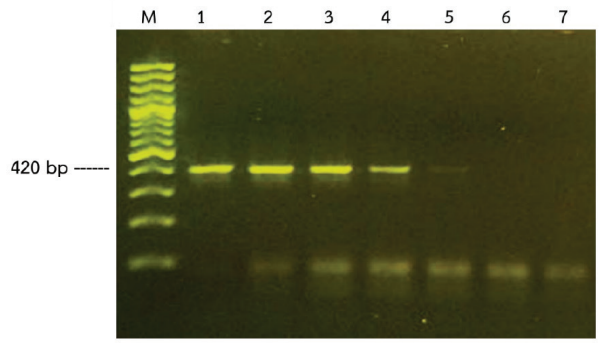


Fig. 4 Sensitivity of the multiplex PCR for detecting of *L. monocytogenes* DNA. Different amounts of *L. monocytogenes* DNA from 1.5 µg to 15 pg was used as template for the multiplex PCR. (lane M: 100 bp DNA marker, lane 1-7: 1.5 µg, 0.15 µg, 15 ng, 1.5 ng, 150 pg, 15 pg, and negative control)

3. Investigation of multiplex PCR in artificially contaminated food

Food samples were added with *L. monocytogenes* or *S. aureus* cells in different concentrations. After enrichment and DNA extraction, the multiplex PCR was able to detect *L. monocytogenes* less than 1 cell/5 g of food (1 cell/g) and 10^5 *S. aureus* cell/5 g of food (2 x 10^4 cells/g) (Fig. 5). Furthermore, there was no amplification for detection of related food pathogens such as *E. coli* and *Vibrio cholera,* represented specificity of the multiplex PCR.

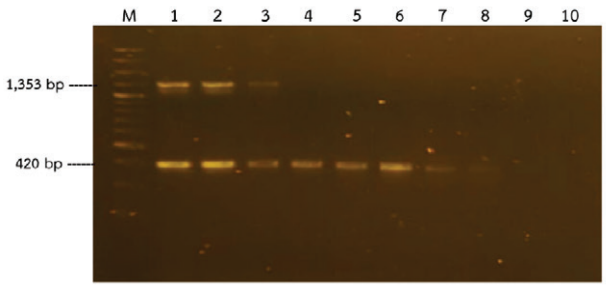


Fig. 5 Sensitivity of the multiplex PCR for detecting of *S. aureus* and *L. monocytogenes* cells in artificially contaminated food. (lane M: 100 bp DNA marker, lane 1: positive control, lane 2-8: 10^6,10^5, 10^4, 10^3, 10^2, 10, 1 cells of each *S. aureus* and *L. monocytogenes*, lane 9-10: negative control)

4. Detection of food samples by using the multiplex PCR

The multiplex PCR developed was used as a tool to detect contaminated foods in local markets. Thirty samples of several types of ready-to-eat foods such as sushi, sausages, undercooked meat, dairy products and refrigerated ready-to-eat foods were randomly purchased and tested. Sample preparation, DNA extraction and detection by multiplex PCR was done as described. The results showed none of them were contaminated with *L. monocytogenes* and *S. aureus.*

Discussion

Contaminated foods represent a risk of infection. Some types of contamination may be caused by infection existing in animals such as products from meat, pork, and chicken. Moreover, food might be contaminated during processing. Contamination of *S. aureus* and *L. monocytogenes* in food is regarded as poor hygiene of food manufacturing. Bacterial identification by conventional method based on culturing and biochemical testing requires several days. Moreover, negative result may be due to presence of antimicrobial agents which can be found in some foods. The development of PCR-based method in this study provides more rapid identification and is able to identify more than one specie.

In this study, *S. aureus* and *L. monocytogenes* were identified using primer for *coa* gene and *prfA* gene, respectively. Coagulase production is a common characteristic for identification of *S. aureus*. *PrfA* gene is and transcriptional activator, required for expression of virulence gene of *L. monocytogenes*. Many studies have developed PCR using various primers (Akineden et al., 2001; Alarcon et al., 2006; Kearns et al., 1999; Li et al., 2008).

Our multiplex PCR showed high sensitivity for detection of *S. aureus* and *L. monocytogenes,* which allowed the detection of 1 ng and 150 pg of the bacterial DNA, respectively. Investigation in artificial contaminated food revealed that 10^4 cell/g of *S. aureus* cells and 1 cell/g of *L. monocytogenes* cells were detected by the multiplex PCR. Infectious dose of *S. aureus* and *L. monocytogenes* is 10^5 - 10^6 cells and 10^3 cells, respectively (Schmid-Hempel & Frank, 2007). Consequently, our multiplex PCR is appropriate to detect those bacteria underneath the range of the infectious doses.

There are many problems when using PCR as a tool for detecting of bacteria directly from foods. They may be due to various factors in foods that can inhibit PCR reaction including inappropriate DNA extraction, low number of bacteria cells, and other unforeseen issues (Aznar & Alarcon, 2003). A variety of substances in foods such as thermonuclease enzyme in milk has been described in inhibiting the PCR and they usually cause false negative results (Wilson et al., 1994). Therefore, increased levels of sensitivity of the PCR are required. Sensitivity of identification of bacteria in food can be improved by a suitable extraction of bacteria. The commercial DNA extraction kit was used in this study, which provided high yield of DNA rather than another method such as the boiling method.

From our investigation, the randomly sampling of ready-to-eat foods in local markets for detecting of *L. monocytogenes* and *S. aureus,* showed that no contaminated foods were detected by using the developed PCR. However, it assessed only pathogens that are mostly found in ready-to-eat foods. Other food-borne pathogens, including *Escherichia coli, Salmonella enteritis, Bacillus cereus, Campylobacter jejuni* and their toxins were not assessed in this study. The determination of ready-to-eat foods in this study was indicated good for microbiological quality, while also revealing a good quality for processing, nevertheless the foods may present preservatives, including acids and salt. Further investigation of contaminated foods should be performed in more samples and locations, which will be adequate to represent food safety in the area.

In many cases of listeriosis outbreaks from foods, the amount of the cells are significantly higher than 1,000 CFU/g (Thevenot et al., 2006). Detection of bacterial contamination in 137 raw food samples from open-markets in Thailand during 2010 to 2011, 5% of samples were contaminated with *L. monocytogenes* and were obtained from meat and fish samples, 39% of samples were

contaminated with *Staphylococcus* spp. which were found in vegetables and fish samples (Ananchaipattana et al., 2012). However, our study investigated the ready-to-eat foods and found no evidence of *S. aureus* or *L. monocytogenes* contamination, indicating a good food processing practice.

Conclusion

The developed multiplex PCR in this study can be used as a rapid, high sensitive and specific method for detection of *S. aureus* and *L. monocytogenes* in contaminated ready-to-eat foods. Investigation of the food pathogens in the ready-to-eat foods also indicates the degree of good hygiene of the foods in these areas. Currently, there are many new techniques that have been developed such as real time-PCR, however multiplex PCR method is still appropriate in laboratory routines and where the other new techniques are not available. Furthermore, multiplex PCR is cost effective and still rapid for the detection. Consequently, this multiplex PCR assay can be further developed for monitoring of *S. aureus* and *L. monocytogenes* contaminated foods. Moreover, it could be a benefit in terms of public health and surveillance of disease outbreaks.

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