DEVELOPMENT OF OLIGONUCLEOTIDE ARRAY FOR DETECTING FOODBORNE PATHOGENS IN FRESH CHICKEN MEAT

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การพัฒนาโอลิโกนิวคลีโอไทด์อเรย์เพื่อตรวจหาเชื้อก่อโรค ในเนื้อไก่สด

นางสาวชนิดา กุประดิษฐ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เทคนิคโอลิโกนิวคลีโอไทด์อเรย์ เป็นเทคนิคที่ใช้การจับคู่เบสอย่างจำเพาะของสายดีเอ็นเอเป้าหมายจากเชื้อ กับดีเอ็นเอติดตามสายสั้น (DNA probes) ที่อยู่บนแผ่นอเรย์ สามารถใช้ตรวจเชื้อก่อโรคทางเดินอาหารจำนวนมากด้วยเวลาอันรวดเร็วในขั้นตอนเดียว การศึกษานี้ได้คัดเลือก ดีเอ็นเอติดตามสายสั้นที่มีความจำเพาะต่อสายดีเอ็นเอเป้าหมาย ให้มีความเหมาะสม เพื่อนำมาใช้ในการตรวจหาแบคทีเรียบ่งชี้คุณภาพด้านความปลอดภัยของเนื้อไก่สดเพื่อการบริโภค โดยติดฉลากสายดีเอ็นเอเป้าหมายจากเชื้อ ด้วยเทคนิคการติดฉลากด้วยสาร digoxigenin (DIG) หลังจากทำการเพิ่มปริมาณของสายดีเอ็นเอเป้าหมาย ซึ่งสามารถตรวจสอบการเกิดการจับคู่เบสอย่างจำเพาะของสายดีเอ็นเอเป้าหมายจากเชื้อกับดีเอ็นเอติดตามสายสั้นที่อยู่บนแผ่นอเรย์ได้ด้วยตาเปล่า ในเบื้องต้นผู้วิจัยได้ใช้ 16S rRNA gene ที่มีความจำเพาะต่อเชื้อ *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes* และ *Clostridium perfringens* เป็นจีนเป้าหมายต้นแบบ ซึ่งพบว่า ความเข้มข้นที่เหมาะสมของดีเอ็นเอติดตามสายสั้นบนแผ่นอเรย์ คือ 200 พิโคโมล การใช้ 16S rRNA gene เป็นจีนเป้าหมายเพียงจีนเดียวนั้น สามารถตรวจเชื้อต่างสปีชีส์กันได้ถึง 5 สปีชีส์ ที่ปริมาณดีเอ็นเอจากจีโนมของแต่ละเชื้อต่ำสุดที่ 1 นาโนกรัม อย่างไรก็ตาม การตรวจหาแบคทีเรียดังกล่าวสามารถทำได้เพียงระดับสกุลเท่านั้น รวมทั้งยังพบการเกิดปฏิกิริยาข้ามกัน กับแบคทีเรียที่ไม่ใช่เชื้อเป้าหมายที่แยกได้จากอาหารจำเพาะที่ใช้เพิ่มจำนวนเชื้อเป้าหมาย อีกด้วย ดังนั้น ผู้วิจัยจึงพัฒนา เทคนิคโอลิโกนิวคลีโอไทด์อเรย์ ร่วมกับเทคนิคที่เพิ่มปริมาณจีนเป้าหมายอื่นที่มีความจำเพาะต่อเชื้อเป้าหมายร่วมด้วย โดยการใช้เทคนิค มัลติเพล็กซ์พีซีอาร์ และพีซีอาร์ดั้งเดิม ในการประเมินผลเทคนิคที่พัฒนาขึ้นมานี้ ใช้เชื้อเป้าหมาย 4 เชื้อ คือ *E. coli*, *L. monocytogenes*, *Salmonella* spp. และ *Shigella* spp. พบว่า สามารถตรวจพบแบคทีเรียเป้าหมายด้วยเทคนิค มัลติเพล็กซ์พีซีอาร์ โดยใช้จีน *usp*A, *prf*A, *fim*Y และ *ipa*H ซึ่งมีความจำเพาะกับเชื้อ *E. coli*, *L. monocytogenes*, *Salmonella* spp. และ *Shigella* spp. ตามลำดับ และหลังจากใช้เทคนิคมัลติเพล็กซ์พีซีอาร์ หรือ พีซีอาร์ดั้งเดิมร่วมกับ เทคนิคโอลิโกนิวคลีโอไทด์อเรย์ ในการตรวจเชื้อเป้าหมาย พบว่า สามารถแยกความแตกต่างของเชื้อเป้าหมายได้ในระดับสกุลและระดับสปีชีส์โดยมีความผิดพลาดในการแปลผลที่ต่ำมาก และเมื่อเปรียบเทียบระหว่างการใช้เทคนิคมัลติเพล็กซ์พีซีอาร์ หรือ พีซีอาร์ดั้งเดิมร่วมกับเทคนิคโอลิโกนิวคลีโอไทด์อเรย์ พบว่า ประสิทธิภาพในการเพิ่มปริมาณ

จีนเป้าหมายด้วยเทคนิคพีซีอาร์ดั้งเดิมนั้นดีกว่า เป็นผลให้สามารถตรวจเชื้อเป้าหมายทั้ง 4 เชื้อได้พร้อมกัน ทั้งในตัวอย่างที่เป็นอาหารเลี้ยงเชื้อบริสุทธิ์ และในตัวอย่างเนื้อไก่สด ในงานวิจัยนี้พบว่าการใช้ เทคนิคมัลติเพล็กซ์พีซีอาร์ และ พีซีอาร์ดั้งเดิม ร่วมกับเทคนิคโอลิโกนิวคลีโอไทด์อเรย์ สามารถตรวจเชื้อทั้ง 4 เชื้อได้ในปริมาณดีเอ็นเอจากจีโนมของแต่ละเชื้อต่ำสุดที่ 1 นาโนกรัม และที่ 0.1 นาโนกรัม ตามลำดับ นอกจากนั้น ยังได้นำเทคนิคนี้ไปประยุกต์ใช้ในการตรวจเชื้อในเนื้อไก่สด 10 ตัวอย่าง ผลการวิจัย พบว่า หลังจากเพิ่มจำนวนเซลล์ของเชื้อเป้าหมายโดยใช้อาหารที่จำเพาะและการเพิ่มปริมาณจีนเป้าหมายโดยใช้เทคนิคพีซีอาร์ดั้งเดิม ร่วมกับ เทคนิคโอลิโกนิวคลีโอไทด์อเรย์แล้ว สามารถตรวจเชื้อได้ทั้ง 4 เชื้อพร้อมกัน ในตัวอย่างเนื้อไก่สด 25 กรัม ในขณะที่การใช้ เทคนิคมัลติเพล็กซ์พีซีอาร์ ร่วมกับเทคนิคโอลิโกนิวคลีโอไทด์อเรย์ สามารถตรวจได้เพียง 3 เชื้อ พร้อมกัน ได้แก่ *E. coli*, *L. monocytogenes*, *Salmonella* spp. จากตัวอย่างเดียวกัน ทั้งนี้ การใช้เทคนิค พีซีอาร์ดั้งเดิม ร่วมกับเทคนิคโอลิโกนิวคลีโอไทด์อเรย์ สามารถใช้ตรวจเชื้อ *Sh. boydii* ที่ปนเปื้อนในเนื้อไก่สด 25 กรัม ที่ระดับความเข้มข้นเริ่มต้นต่ำสุดอย่างน้อย 3 เซลล์ และ *L. monocytogenes* ที่ระดับความเข้มข้นเริ่มต้นต่ำสุดอย่างน้อย 10 เซลล์ขึ้นไป

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

CHANIDA KUPRADIT : DEVELOPMENT OF OLIGONUCLEOTIDE ARRAY FOR DETECTING FOODBORNE PATHOGENS IN FRESH CHICKEN MEAT. THESIS ADVISOR : ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 201 PP.

OLIGONUCLEOTIDE ARRAY/FOODBORNE PATHOGENS/DNA PROBES

Oligonucleotide array hybridization based methods can be used for screening of multiple foodborne pathogens. Several target pathogens can be monitored in a single step of DNA hybridization using suitable specific probes on an array matrix. In this investigation, screenings of suitable probes for specific detection of foodborne pathogens prevalence in fresh chicken meat were performed using post-PCR labeled target regions. The hybridization signals of non-radioactive labeling digoxigenin (DIG) incorporated into the PCR target regions were observed by naked eyes. The target regions of 16S rRNA gene specific for *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, and *Clostridium perfringens*, were used as models. The optimum concentration of the oligonucleotide probes was found to be 200 pmol. The detection using only 16S rRNA gene as target gene was carried out to detect multiple target bacteria at as low as 1 ng in the mixed genomic DNA from the 5 bacterial species. Although the results showed that a large number of target bacteria can be detected with easy result interpretation by oligonucleotide array hybridization but some of them can be differentiated in only the genus level and some cross-reactivities were found from the non-target bacteria isolated from the enrichment culture. Therefore, oligonucleotide array combined with multiplex PCR (m-PCR) or conventional PCR using specific genes as targets were developed to specifically

detect dominant foodborne pathogens in chicken meat. Target bacteria including *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. were used as models for the evaluation of these combined methods. M-PCR targeting the *usp*A, *prf*A, *fim*Y, and *ipa*H was successfully used to detect *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp., respectively. The combination of m-PCR or conventional PCR with oligonucleotide array revealed discriminatory power among genera and species of *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. with low or no incident of false negative results. The efficiency of the conventional PCR amplification is more sensitive than that of m-PCR for amplification of target genes. The m-PCR- and conventional PCR-oligonucleotide array could detect all 4 target bacteria at as low as 1 ng and 0.1 ng of each in the mixed genomic DNA extracted from pure cultures, respectively. The application of oligonucleotide array was tested with 10 fresh chicken meat samples. Combination of target bacterial enrichment and DNA amplification demonstrated that the conventional PCR-oligonucleotide array could be used for simultaneously detection of all 4 target bacteria in fresh chicken meat samples while m-PCR-oligonucleotide array could simultaneously detect only *E. coli*, *Salmonella* sp., and *L. monocytogenes* in the same samples. Conventional PCR-oligonucleotide array was able to detect *Sh. boydii* and *L. monocytogenes* at initial concentration of at least 3 and 10 cells in 25 g sample, respectively.

School of Biotechnology

Academic Year 2012

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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CHAPTER I

INTRODUCTION

In Thailand, poultry especially frozen and processed chicken meat is one of the most important foods for exports. Thailand has been one of the major poultry export country in the world. In 2003, Thai exported processed chicken to Japan and European Union (EU) approximately 13.9% and 19.7% of all food exports, respectively (Food intelligence center Thailand, 2009). In 2008, Thai exported approximately 50,275 million baht of processed chicken which is 25% of the world market (http://fic.nfi.or.th). The export of frozen and processed chicken during the first three month of 2009 increase in values approximately 7.9%, (www.depthai.go.th). Thailand's exports of processed chicken meat are forecasted to grow by 8-10% in 2009 in anticipation of continued strong demand from the EU and Japan (http://www.thepoultrysite.com). In 2011, chicken meat exports (both cooked and uncooked) increased 10% in quantity to 217,906 metric tons from the same period of 2009, while the value of exports increased by 22% in the first half of 2011. Both domestic consumption and exports in 2012 are also forecast to grow 9% reflecting strong consumer demand (USDA Foreign Agricultural Service, http:// www. thepoultrysite. com/ articles/ 2165/ thailand-poultry-and-products-annual-2011). Thus Thailand’s total cooked and frozen chicken production is predicted to increase further in line with growing domestic consumption and export. Poultry meat can be contaminated with foodborne pathogens due to many factors such as nutrients,

high water activity, and neutral pH. These factors are favorable conditions for the development of contaminated microorganisms from external sources during processing (Guerra, 2009). Thus foodborne pathogen detection prior to export is very important to increase the confident of the international markets in term of chicken meat quality from Thailand.

In EU, the prevention of human health from hazardous unsafe chicken meat has been reported in 'European Legislation in Relation to Food Safety'. The main regulations focus on *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* (National food institute, 2008). In Japan, the control of foodborne pathogen in frozen, fresh, and processed chicken has been reported in 'The food sanitation law'. For fresh and processed chicken meat, the limitations of contaminated foodborne pathogens focuses on *E. coli* (absence in sample), *Staph. aureus* (<1,000 colonies in 1 g sample), *Salmonella* spp. (absence in sample), *Clostridium* spp. (<1,000 colonies in 1 g sample of processed chicken), and coliform group (absence in sample) (National food institute, 2009). In Thailand, Department of Livestock Development regulated contaminated bacterium level for exported meat product as described in 'Microbiological Guideline for Chilled/ Frozen Meat and Poultry Meat' and 'Microbiological Standard for Livestock Products'. For chilled/ frozen poultry meat', the limitations of contaminated foodborne pathogens focuses on coliform (<5,000 org/g), *E. coli* (<100 org/g), *Staph. aureus* (<100 CFU/g), *Enterococci* spp. (<1,000 CFU/g), *Salmonella* spp. (absence in 25 g sample), *L. monocytogenes* (should be absence in 25 g sample), *Campylobacter jejuni*, and *Camp. coli* (should be absence in 25 g sample). For heat-treat meat products and reheat after packing, the microbial regulation mainly focuses on coliform (absence in 1 g sample), *E. coli* (absence in 1 g

sample), *Staph. aureus* (absence in 1 g sample), *Enterococci* spp. (<100 CFU/g), *Salmonella* spp. (absence in 25 g sample), *L. monocytogenes* (absence in 25 g sample), *Camp. jejuni*, and *Camp. coli* (absence in 25 g sample) (Department of livestock development, 2009).

Foodborne pathogens including *Campylobacter* spp., *Clostridium perfringens*, *L. monocytogenes*, *Salmonella* spp., *Staph. aureus*, and microbial food safety indicator, *E. coli*, which are prevalence in chicken meat, should be monitored prior to export. Therefore, the developments of accurate and rapid methods for foodborne pathogens detection were considered in this research. Common method of detection is the culture based test, which utilizes suitable growth media to identify bacterial species, thus between 24-48 h are needed to obtain the results (Yoo et al., 2004). Numbers of apply research in this field have been interested in rapid methods for pathogen detection. These methods include antibody-based assays, genetic amplification methods, and oligonucleotide array.

Genetic amplification method such as the polymerase chain reaction (PCR) has been applied due to its rapidity time and high level of specificity. This technique is able to distinguish closely related species that most antibody tests could not (Nugen and Baeumner, 2008). In complex mixture contained various microorganism communities, large numbers of primers are needed for multiple pathogen detection. Multiplex PCR (m-PCR) is an effective way for numbers of microbial detection but the detection sensitivity for some target bacteria decreases. Disadvantages of this technique involve the chance that two unrelated primers produce spurious product increases (Chiang et al., 2006). Moreover, the detection of PCR and m-PCR

amplicons is based on the separation of PCR products using different molecular weight by agarose gel electrophoresis which is less sensitive and sequencing are needed for PCR validation step (Chiang et al., 2006; Settanni and Corsetti, 2007; Wang et al., 2007). Recently, real-time PCR has been applied to improve the sensitivity of m-PCR and PCR techniques. However, real-time PCR requires special thermal cyclers, fluorescent detectors to detect several m-PCR products and expensive reaction reagents (Bai et al., 2010; Suo et al., 2010, Hu et al., 2011). Therefore, simple and rapid methods with inexpensive equipment for data analysis are needed for PCR validation step for simultaneous multiple pathogen detection.

To solve these problems, oligonucleotide array can be applied in a single step for several pathogen detection. Recently, DNA and oligonucleotide array has been applied as a tool for sensitive and high-throughput multiple organism screening (Yoo et al., 2004). These oligonucleotide probes can be designed to hybridize to different groups of bacterial species using variable target sequence regions. Targets for hybridization may be PCR products, oligonucleotide, genomic DNA, or cDNA (Franke-Whittle et al., 2006). To improve the sensitivity of hybridization technique, amplification of target prior to hybridizing with oligonucleotide array has been suggested (Chiang et al., 2006). Although microarray-based techniques have several advantages, but the regular microarray methods need expensive equipments for array development, array scanning and data collection (Bai et al., 2010), which is beyond the budget of many laboratories especially in developing countries. Thus the development of signal investigation system with easy, low cost but high sensitivity has been required. Easy systems for hybridization signal detection and result interpretation from oligonucleotide array using immunological chromogenic reaction

which can be observed by naked eyes were applied in this research.

The aim of this investigation focused on development of multiple foodborne pathogen detection in fresh chicken meat by oligonucleotide array hybridization technique using *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. as model organisms. Moreover, *Staph. aureus* and *Cl. perfringens*, the regulated foodborne pathogens in poultry meat, were also differentiated by oligonucleotide array based method. The primers and probes were designed using both conserved and specific genes as targets since most of the research also reported the limitation of 16S rRNA regarding to its diversity. All primers and probes from this research were tested for specificity using both references and isolated bacterial strains of chicken intestinal tracts including target and non-target bacteria which are frequently found in enrichment culture. Target regions for DNA labeling were amplified by conventional PCR or m-PCR followed by oligonucleotide array hybridization. Easy systems for hybridization signal detection using immunological chromogenic reaction which can be observed by naked eyes were performed. Efficiency and detection limit of these systems for multiple target bacterial detection were evaluated in pure culture and in real fresh chicken meat samples.

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CHAPTER II

FOODBORNE PATHOGEN ISOLATIONS

Abstract

There are numerous numbers of commercial chicken farms in Thailand but lack information of the isolation and characterization of chicken intestinal foodborne pathogens and food safety indicators. Therefore, isolation of food safety indicators and foodborne pathogens include *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Clostridium perfringens*, and *Campylobacter* spp. from 5 fresh chicken intestines of 4 different farms in Nakhon Ratchasima, Thailand were attempted. Contaminations of *L. monocytogenes* and *Campylobacter* spp. were not found from all tested chicken intestines. Only *Cl. perfringens*, *E. coli*, and *Salmonella* spp. were found in this investigation. Several isolates of *E. coli*, *Cl. perfringens* and *Salmonella* spp. were identified by biochemical reactions. Minor variation of biochemical profiles especially carbohydrate utilization and gelatin hydrolysis were found among isolates. Identification of some non-target bacteria isolated from enrichment culture using only biochemical profiles were difficult. The target and non-target isolates which have physiological characteristic diversity observed in this part were used as the tested organisms for the development of foodborne pathogen detection to increase the specificity and accuracy of the novel rapid methods in next part.

2.1 Introduction and review literature

2.1.1 Poultry microflora

Several hundred different species of microorganisms from poultry meat have been reported. Microorganisms found on poultry can be divided into two general groups. Firstly microorganisms that can produce disease in humans, generally referred to as pathogens, and those not associated with a recognized disease which are designated as non-pathogenic organisms (Mountney and Parkhurst, 1995). More than 40 different types of anaerobes Gram-negative and Gram-positive non-sporing rods and cocci have been isolated from chicken caeca. The microflora of the chicken crop consists of large numbers of lactobacilli, smaller numbers of coliforms, and streptococci. The dominant lactobacilli in the crop is maintained by their ability to adhere to the crop epithelial cells (Fuller, 2001). Coliforms are a large group of Gram-negative, facultative anaerobic, non-spore-forming, rod-shaped bacteria that all belong to a single taxonomic family Enterobacteriaceae. They differ from most of the other members of this family because of their ability to ferment lactose, with the production of acid and gas occurring within 48 h. Most coliforms are present in large numbers among the intestinal flora of humans and other warm-blooded animals, and are thus found in fecal waster (Rompré et al., 2002; Paruch and Mæhlum, 2012). Coliforms are comprised of several genera such as *Klebsiella*, *Escherichia*, and *Enterobacter*. However, *E. coli* and *Enerobactert aerogenes* are the most widely recognized members of these genera. The detection of large numbers of these organisms in foods and water may be indicator of fecal pollution or contamination. The present of coliforms in large numbers in processed food indicates poor practice in the processing, and storage of food which raises the possibility that pathogens might

have entered the food product through the same route. One of the important bacterial intestinal tracts is enterococci. The enterococci are members of the genus *Streptococcus*, which are Gram-positive, catalase negative cocci in short or long chains. *Streptococcus faecalis* and its subspecies are more commonly associated with the intestinal tract of human. The enterococci are a better index of food sanitary quality than are coliforms especially for frozen foods (Chipley, 1987).

The small intestine microfloras in young chicks are mainly facultative anaerobes (*Streptococcus*, *Staphylococcus*, *Lactobacillus*, and *E. coli*). The main types of *Lactobacillus* in germ-free chicken reviewed by Barnes (1979) are *Lact. acidophilus*, *Lact. salivarius*, and *Lact. fermenti*. However, large numbers of anaerobes such as *Eubacterium*, *Propionibacterium*, and *Clostridium* have been isolated from the duodenum and ileum (Smirnov et al., 2004). Currently many different chicken probiotics are lactic acid bacteria. They may contain only one strain such as *Lact. reuteri* or as many as several stains such as *Lact. acidophilus*, *Lact. delbrueckii* subsp. *bulgaricus*, *Lact. plantarum*, *Lact. rhamnosus*, *Enterococcus faecium*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum* (Fuller, 2001). The psychrophilic bacteria belonging to the genera *Flavobacterium*, *Acinetobacter*, and *Corynebacterium* have been isolated from live chickens at processing plants. These psychrophilic bacteria are present on the feet, feathers, and skin of live birds but their numbers decrease markedly after scalding (Bailey et al., 1987).

2.1.2 Foodborne pathogens in poultry

Pathogenic organisms can be divided further into two groups which can produce disease by invading the body and producing an infection such as *Salmonella*, Streptococci, and pathogens which produce toxins or poisons in the food itself such as

Staphylococcus and *Clostridium*. Another basis for classifying pathogens in or on meat is enteric diseases or those that reside in digestive tract, extraintestinal illnesses from foodborne infectious agents, and occupational diseases transmitted to workers who handle animals and animal products. *Salmonella*, *Campylobacter* spp. and *Clostridium perfringens* are examples of the first type. *Clostridium botulinum* and *Staph. aureus* are example of the second type and *Chlamydia psittaci* is one example of an occupational infectious disease (Mountney and Parkhurst, 1995). In chicken meat, foodborne pathogen including *Salmonella* spp., *L. monocytogenes*, *Staph. aureus*, *Camp. jejuni*, *Camp. coli*, and *E. coli* have been regulated by food regulation law in Thailand (Department of livestock development, 2009). The processes of rinsing and processing poultry can introduce varieties of potential sources of pathogen contamination. A processed carcass may contain pathogens on the skin that are attached by specific or non-specific interaction, entrapped in folds, crevices, pores (follicle) or water-skin interface (Mandrel and Wachtelt, 1999).

Listeria monocytogenes is a facultative small short, Gram-positive rod bacterium. It has a temperature growth range of 2.5 - 44^{o}C and it is capable of causing serious illness in human and animals. *Listeria* is flagellated and motile in a characteristic tumbling or slightly rotating fashion. The productions of flagella are regulated by temperature. Motility is best demonstrated at 20^{o}C. Growth along the stab line in an appropriate medium showed spreading 3-5 mm below the surface of the medium in an umbrella fashion that may be seen as early as 24 h (Lovett, 1989). The pathogen is widely distributed in the environment. Its primary habitat may be soil and decaying vegetation. It has the ability to grow at refrigeration temperatures and to tolerate a wide range of pH and osmolarity. Infection is commonly induced by

contaminated food especially cold-stored food. Infection has been associated with a variety of foods, including cheese, meat, milk, vegetables and fish. The infection risk of this pathogen are pregnant women, newborn babies, elder, and immune compromised persons (Kathariou, 2000; O'Grady et al., 2009). The prevalence of *Listeria* in poultry has been reported in many publications. In 2001, Capita and colleagues reported the prevalence of *Listeria* spp. on the skin of a hundred fresh eviscerated and refrigerated chicken carcasses purchased from 20 retail stores in Leo´n (Spain) using a two-stage enrichment procedure similar to the USDA method. They found that 95% of poultry skin samples contained *Listeria* spp., 15% of these contained *L. monocytogenes*, 17% *L. monocytogenes* and other *Listeria* species in combination, and 63% of the samples being contaminated by other *Listeria* such as *L. innocua*, *L. welshimeri*, *L. grayi* and *L. ivanovii* (Capita et al., 2001). In Thailand, small numbers of *L. monocytogenes* have been isolated from chicken in open markets (6%) and supermarkets (4%) in Bangkok and Pathum Thani provinces (Minami et al., 2010). In 2010, Stonsaovapak and Boonyaratanakornkit investigated the prevalence of *Listeria* spp. from meat and meat products, dairy and dairy products, fresh vegetables, fresh seafood, and ready-to-eat food collected from supermarkets in Bangkok, Thailand. The prevalence of *Listeria* spp. was 16.8%, most of them were isolated from raw meat and vegetables. *L. monocytogenes* was isolated from 18 out of 380 (4.7%) studied samples. Other species isolated were *L. innocua* (6.6%), *L. ivanovii* (0.8%), *L. seeligeri* (0.5%), *L. grayi* (1.6%) and *L. welshimeri* (2.6%) (Stonsaovapak and Boonyaratanakornkit, 2010).

The genus *Campylobacter* is Gram-negative, slender, curved bacteria that are motile by a single, polar flagellum. Growth will occur between 25 and 43^{o}C.

Camp. jejuni is an obligate microaerophile grows optimally in an atmosphere containing 5% oxygen (Stern, 1989). *Camp. jejuni* and *Camp. coli* are the most common poultry pathogens that cause human gastrointestinal infections. The handling and ingestion of contaminated poultry products are risk factors for sporadic campylobacteriosis (Lilja and Hänninen, 2001). In Thailand, the majority (52%) of *Campylobacter* isolates from chickens were *Camp. coli* (Padungtod and Kaneene, 2005). In 2005, the contaminations of *Campylobacter* were reported in food animal and humans in Chaing Mai and Lampang, provinces of northern Thailand, from 2000 to 2003. A total of 2,360 samples were processed by cross-sectional study. Their results indicated that the prevalence of *Campylobacter* in chickens at the farms, slaughterhouses, and markets were 64, 38, and 47%, respectively. In 2007, Vindigni and colleagues investigated the prevalence of *Salmonella*, *Campylobacter*, *Acrobacter*, and *Enterococcus* on raw beef, chicken meat, pork, and chicken eggs from supermarket and fresh market in Bangkok. They reported that the contamination of *Salmonella* spp., *Camp. jejuni* and *Camp. coli* in chicken samples (50 samples) were 62%, 24%, and 28%, respectively.

Salmonella spp. are facultative anaerobic, Gram-negative, straight, rods, which are usually motile with peritrichous flagella. *Salmonella* spp. causes illness by means of infection. They multiply in small intestine, colonize and subsequently invade the intestinal tissue, producing an enterotoxin and causing an inflammatory reaction and diarrhea. The organism can get into the blood stream and/or the lymphatic system and cause more severe illness (Bell and Kyriakides, 2002). This organism is an important bacterial pathogen in human and animal including cows, pigs, chickens, and turkeys. In the United States, drug resistant *Salmonella* serotype

Typhimurium (*S.* Typhimurium) was the most commonly isolated serovar (29.4%) from 1968 to 1998 (Courtney et al., 2006). Several research have reported that *Salmonella* can attach to wide range of inert surfaces such as steel, glass and polymer substrate, and to biological surfaces such as skin, muscle and cell membrane (Cloak et al., 1999). In Thailand, *Salmonella* isolated from poultry have been reported. Bangtrakulnonth and colleagues found 44,087 *Salmonella* serotypes isolated from humans and 26,148 from other sources in Thailand between 1993 through 2002. They showed that the most common serovar causing human salmonellosis in Thailand was *Salmonella enterica* Weltevreden. In frozen chicken, *S.* Enteritidis (19.9%), *S.* Hadar (9.3%), *S.* Paratyphi B var Java (7.1%), *S.* Virchow (5.9%), *S.* Blockley (4.6%), *S.* Schwarzengrund (3.9%), *S.* Agona (3.1%), *S.* Anatum (2.9%), *S.* Amsterdam (2.5%), *S.* Emek (2.5%) can be isolated. Their results indicated that serovars causing human infections in Thailand seem to be related to *Salmonella* serovars in different food products and reservoirs (Bangtrakulnonth et al., 2004). In 2005, Angkititrakul and colleagues isolated *Salmonella* from 40 samples of chicken meat which were collected from retail markets in Khon Kaen, a province in northeast Thailand between January and December 2003. They found that the most prevalent serovar was *S.* Anatum (33.3%), followed by *S.* Rissen (16.7%), *S.* Virchow (13.3%), *S.* Enteritidis (13.3%), *S.* Agona (10%), *S.* Derby (10%), *S.* Worthington (3.8%), and *S.* Panama (3.3%) (Angkititrakul et al., 2005).

For *E. coli*, this organism was found to be a dominant bacterium of the facultative anaerobic normal flora of the intestine of warm blood animals, and was shown to play an important role in maintaining intestinal physiology. *E. coli* is Gram-negative, straight rods that may be peritrichously flagellated or non-motile.

Generally, *E. coli* strains that colonize the human bowel are harmless commensals. However, within the species there are fully pathogenic strains that cause distinct syndromes of diarrheal disease. These diarrheagenic *E. coli* are grouped into four categories. The four main categories include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and enterohemorrhagic *E. coli* (EHEC) or *E. coli* O157:H7 (Doyle and Padhye, 1989). In 2006, Akkaya and colleagues reported that poultry meat can also be a source of *E. coli* O157:H7 infections for humans. They determined the prevalence of *E. coli* O157:H7 on various portions of chicken carcasses obtained from retail markets and poultry shops in Turkey using immunomagnetic separation methods. The results showed that *E. coli* O157:H7 can be isolated from two (1.05%) of the 190 samples of poultry meat examined which may result either from cross-contamination during slaughter, and/or processing or during transportation (Akkaya et al., 2006).

The genus of *Shigella* is a member of the family Enterobacteriaceae classified into four groups as follows: *Shigella dysenteriae* (group A), *Sh. flexneri* (group B), *Sh. boydii* (group C), and *Sh. sonnei* (group D). The bacterium *Shigella* was identified as cause of diarrheal disease in human. All groups cause disease in humans, although with some differences in clinical spectrum. Clinical manifestations of classic bacillary dysentery include fever, vomiting, abdominal pain, tenesmus (painful straining to pass stool). The stool usually contains blood, mucus, and inflammatory cells which result from invasion of the pathogen into the intestinal mucosa. The Shigellae are non-motile, usually anaerogenic Gram-negative bacteria that do not ferment lactose or ferment it slowly. Shigellae are generally considered to compete poorly with other enteric flora. However, when experimentally inoculated

into food samples in high numbers, *Shigella* may survive for periods ranging from less than 3 weeks to more than 3 months in such diverse food as stewed apples, cheese, flour, milk, seafood, eggs, tomato juice, cooking oil, root beer, and ginger ale. Unlike most other enteropathogenic bacteria, *Shigella* strains are known to cause adult infections at a dose of 10^1-10^2 organisms. Because of most cases of shigellosis are spread by person-to-person transmission, individuals most frequently affected are those with poor personal hygiene, such as young children, people in custodial institutions, and persons in lower socioeconomic groups subject to crowded living conditions, inadequate water supplies, and poor sanitation systems (Wachsumuth and Morris, 1989; Pulsrikarn et al., 2009).

In Thailand, Chanachai and colleagues (2008) investigated a foodborne outbreak of gastroenteritidis due to *Shigella* and possibly *Salmonella* in a school. The outbreak evidence related to two enteric pathogens including *Sh. sonnei* and *Salmonella* spp. Among stools samples from 103 cases, *Shigella* group D was found in 18 cases, *Salmonella* group C in 5 cases, and *Salmonella* group E in 2 cases. However, they could not conclude that the contamination was not introduced by an infected food handler, since *Shigella* was not recovered from any of the food handlers (Chanachai et al., 2008). Pulsrikarn and colleagues investigated species and serotypes of *Shigella* isolated from clinical samples in Thailand from 2001 to 2005. They reported that *Sh. sonnei* was the most common species isolated, consisting of approximately 80% of all *Shigella* spp. each year, while *Sh. dysenteriae* and *Sh. boydii* were uncommon (Pulsrikarn et al. 2009). In 2010, Minami and colleagues investigated the prevalence of *Shigella* in shrimp (26 samples) and oyster (5 samples) collected from open markets and supermarkets in Thailand. They reported that no

contaminations of *Shigella* were observed from these food samples (Minami et al., 2010). These results indicated very low prevalence of *Shigella* from food samples in Thailand. However, studies on DNA relatedness showed that these four *Shigella* species and *E. coli* are closely related genetically and could be considered to be in the same species (Wachsumuth and Morris, 1989). Therefore, all 4 species of *Shigella* were used as the tested organisms in our investigation because these bacteria might cross-reactivity with *E. coli* in the samples.

Clostridium perfringens is the most extensively studied anaerobic bacterium that is pathogenic to human. *Cl. perfringens* is a typical Gram-positive, spore-forming, rod-shaped bacterium that is encapsulated and non-motile. The organism produces several biologically active proteins; some are toxins and some are enzymes. The strains are classified into five types, A-E, on the basis of production of four extracellular toxins: alpha, beta, epsilon, and iota. Food poisoning due to *Cl. perfringens* usually occurs 8-24 h after the ingestion of food containing large number of vegetative cells. Diarrhea and severe abdominal pain are the usual symptoms. Nausea is less common but fever and vomiting are unusual. In a very few isolated cases, food poisoning symptoms appeared within 2 h. This suggests the role of a preformed toxin or ingestion of some meat products. Intoxication with *Cl. perfringens* can be caused by ingestion of food containing $\geq 10^5$ CFU/g of an enterotoxigenic strain. In vivo production of the enterotoxin is associated with sporulation in the intestine. Enterotoxin formation in meat and poultry items has also been shown to occur. The widespread of *Cl. perfringens* have been found in raw or frozen meat and poultry (ranging of 30-80%). Thus, meat and poultry are the most common vehicles of *Cl. perfringens* food poisoning (Labbe, 1989; Aguilera et al., 2005).

The prevalence of these harmful pathogenic bacteria in poultry meat has been reported therefore, rapid and high sensitivity method for pathogen detection is required. The specificity of detection by developed techniques depends on the target gene of interest and the bacterial background from the sample. However, the investigations of specificity of target genes in isolated strains in Thailand are still limited. To improve the specificity of the molecular based method in this research, several isolated strains of the target bacteria were tested.

In Thailand, there are numerous commercial chicken farms but information of the isolation and characterization of chicken intestinal foodborne pathogens and food safety indicators are still lacking. The investigations of foodborne pathogens dominated in chicken intestine are good index of chicken farms sanitation. In this investigation, chicken intestine was chosen as the source for bacterial isolation based on its diversity of intestinal microflora. Chicken gastrointestinal tract is the major digestive and absorption organ. A surprisingly diversed microbiota has been found throughout the tract and is most extensive in the cecum. Most poultry foodborne pathogens are found in the intestine (van der Wielen et al., 2002; Lu et al., 2003; Amit-Romach et al., 2004). The diversity of physiological characteristics of the foodborne pathogens obtains from this chapter were used as the tested organisms for the development of rapid methods for foodborne pathogen detection in Chapter 3 and 4.

2.1.3 Objective of foodborne pathogen isolation

Isolation of foodborne pathogens and microbial food safety indicators include *E. coli*, *Campylobacter* spp., *Salmonella* spp., *Cl. perfringens*, and

L. monocytogenes from chicken intestines of different commercial farms were performed. The physiological characteristics of each isolate were performed.

2.2 Materials and methods

2.2.1 Sample collections

A total of 5 fresh chicken intestine samples were collected from 4 different farms located in Nakhon Ratchasima, Thailand between April and August 2010.

2.2.2 Target bacterial isolation and biochemical reactions identification

Modifications of methods described in Bacteriological Analytical Manual (United States Food and Drug Administration, 1998) were used to isolate *E. coli*, *Campylobacter* spp., *Salmonella* spp., *Cl. perfringens*, and *L. monocytogenes*. Chicken intestines were cut and placed into stomacher bags. Ten volume of appropriate pre-enrichment broth was added into each sample and homogenized by a laboratory blender stomacher 400 (Seward Laboratory System Inc., New York, USA). Pre-enrichment and enrichment steps were performed as described below. The target bacteria and the non-presumptive colonies were collected and identified by biochemical reactions.

2.2.2.1 *Campylobacter* spp.

Initial step of pre-enrichment was performed by incubating samples in 4 volume of Bolton broth based supplement with 5% lysed horse blood and Bolton antibiotic additive (OXIOD, Basingstoke, Hampshire, England). Samples were homogenized by stomacher machine at low speed for 1 min. The medium were transferred to sterile loosely caped flasks and incubated at 37^{o}C for 4 h under

microaerophile condition created by Campy pack (OXIOD). After 4 h, the enrichment step was performed by incubate the pre-enrichment culture at 42^{o}C for 48 h under microaerophile condition. Enrichment culture broth was streaked and spread on *Campylobacter* blood-free selective agar (Modified CCDA-PRESTON) supplement with CCDA selective supplement (OXIOD) and incubated at 37^{o}C for 48 h under microaerophile condition. The presumptive and non-presumptive colonies observed on mCCDA were re-streaked on mCCDA medium. The screening of *Campylobacter* bacteria were done under both microaerophile and standard condition. Only non-*Campylobacter* bacteria were able to grow under standard and microaerophile conditions. Non-*Campylobacter* bacteria were tested for biochemical characteristic properties as described in the *Salmonella* spp. and *E. coli* isolation (2.2.2.3 and 2.2.2.5).

2.2.2.2 *Clostridium perfringens*

For isolation of *Cl. perfringens*, 10 volume of peptone dilution fluid (Appendix I, M1.4) was added into sample and homogenized at normal speed for 1 min. The homogenate was serially diluted into peptone dilution fluid and 100 µl of each dilution was spread on tryptose sulfite cycloserine agar (TSC) (Biomark, Pune, India) containing antibiotic additive and egg yolk emulsion (Biomark). The cultivation was overlaid with TSC agar based and incubated under anaerobic condition at 37^{o}C for 24 h. The presumptive colonies were tested for biochemical properties as described by Bacteriological Analytical Manual (United States Food and Drug Administration, 1998) including motility, lactose fermentation, catalase test, gelatin liquefactions, nitrate reduction and stormy fermentation.

2.2.2.3 *Escherichia coli*

For isolation of *E. coli* and coliform bacteria, 10 volume of Butterfield's phosphate-buffered water (Appendix I, C3.1) was added into sample and homogenized at normal speed for 1 min. One milliliter of homogenized solution was transferred to lauryl tryptose broth (LST) (Appendix I, M1.3) and incubated at 37^{o}C for 24 h. One loopful of gassing LST culture were inoculated in brilliant green lactose, bile 2% (BGLB) (OXIOD) and incubated at 37^{o}C for 24–48 h. Gassing BGLB culture were streaked and spread on eosin-methylene blue (EMB) agar (Himedia, Mumbai, India) and incubated at 37^{o}C for 24-48 h for *E. coli* and coliform bacteria isolation. The typical and untypical *E. coli* colonies were subcultured on EMB (Himedia) and MacConkey agar (Himedia) and incubated at 37^{o}C for 24 h. Single colony was re-streaked on TSA (Appendix I, M1.6) and tested for biochemical characteristic as described for the *Salmonella* isolation part (2.2.2.5).

2.2.2.4 *Listeria monocytogenes*

Pre-enrichment step was performed by adding 10 volume of half-Fraser broth (OXIOD) into sample and homogenized at normal speed for 1 min. The homogenate was transferred to a sterile flask and incubated at room temperature for 24-48 h. Then 100 µl of the culture broth was transferred into 10 ml of Fraser broth (OXIOD) and incubated at 37^{o}C for 24 h. The culture broth was streaked and spread on PALCAM agar with and without antibiotic supplement (OXIOD) and incubated at 37^{o}C for 48 h. The suspected and unsuspected-*Listeria* colonies were subcultured on TSA and incubated at 37^{o}C for 24-48 h. Colonies of presumptive *Listeria* sp. on TSA were collected and submitted for Gram stain and identification by biochemical characteristic tests (Appendix II) including, oxidase, catalase, urea hydrolysis,

motility, carbohydrate utilization, H_2S production, Indole production, Voges-Proskauer (VP) reaction, Methyl red reactive compound test (Cappuccino and Sherman, 1999; United States Food and Drug Administration, 1998). All non-*Listeria* bacteria were tested for biochemical characteristic as performed for *Salmonella* and *E. coli*.

2.2.2.5 *Salmonella* spp.

Pre-enrichment culture was performed by adding 10 volume of lactose broth (LB) (Appendix I, M1.2) into sample and homogenized at normal speed for 1 min. The cultures were incubated at 37°C for 24 h. The enrichment steps were initiated by transferring 100 µl of pre-enrichment culture each to 10 ml Rappaport-Vassiliadis (RV) broth (Himedia) and 10 ml tetrathionate (TT) broth (Himedia) and incubated at 42°C for 24 h. Then the culture broth were streaked and spread on xylose lysine desoxycholate (XLD) agar (OXIOD) and incubated at 37°C for 24 h. Typical *Salmonella* and non-*Salmonella* colonies were subcultured on bismuth sulphite (BS) agar (OXIOD) and incubated at 37°C for 24 h. For single colony purification, the suspected colonies showing typical-*Salmonella* and non-*Salmonella* morphologies were re-streaked on trypticase soy agar (TSA) and tested for Gram stain and biochemical characteristics (Appendix II). The biochemical reactions for *Salmonella* identification were oxidase, catalase, urea hydrolysis, motility, gelatin hydrolysis, nitrate reduction, carbohydrate utilization, H_2S production, IMViC (Indole production, Methyl red reactive compound test, Voges-Proskauer (VP) reaction, and Citrate test) (Cappuccino and Sherman, 1999; United States Food and Drug Administration, 1998).

2.3 Results

2.3.1 Isolation of target bacteria and identification by biochemical reactions

The occurrence of 4 foodborne pathogens and 1 food safety indicator were investigated from 4 different farms located in Nakhon Ratchasima, Thailand as summarized in Table 2.1. Of 5 chicken intestines from 4 farms, no *L. monocytogenes* nor *Campylobacter* spp. were detected. However, *L. innocua* was detected from sample 2 from farm A. *E. coli* and *Cl. perfringens* were detected when specifically isolated but *Salmonella* sp. was detected from sample 1 from farm A only.

Table 2.1 Foodborne pathogens and microbial food safety indicators isolated from 4 samples of chicken intestines

Sample No.	**Farm**	**Bacteria isolation**	**Bacteria detected**
1	A	*Campylobacter* spp. *Cl. perfringens* *E. coli* *L. monocytogenes* *Salmonella* spp.	*Cl. perfringens* *E. coli* *Salmonella* sp.
2	A	*Campylobacter* spp. *L. monocytogenes*	*L. innocua*
3	B	*Campylobacter* spp. *L. monocytogenes*	Not detected

Table 2.1 (Continued)

Sample No.	**Farm**	**Bacteria isolation**	**Bacteria detected**
4	C	*Campylobacter* spp. *L. monocytogenes*	Not detected
5	D	*Cl. perfringens* *E. coli* *L. monocytogenes* *Salmonella* spp.	*Cl. perfringens* *E. coli*

2.3.1.1 Isolation of *Campylobacter* spp.

In this investigation, *Campylobacter* was screened from only 4 samples. However, no *Campylobacter* spp. was found. More than 10 colonies were re-streaked on mCCDA agar and incubated under standard and microaerophilic conditions. All isolates were able to grow under both standard and microaerophilic conditions. These results indicated that all isolates observed were not *Campylobacter* bacteria indicated that no *Campylobacter* spp. from 4 chicken intestines from Nakhon Ratchasima was detected. However, 2 of the non-*Campylobacter* bacteria isolates were randomly chosen (CM2 and CM7) and biochemical characteristics were identified (Table 2.5). Both isolates had the biochemical profiles similar to *Salmonella* sp. except for no gas production from myo-inositol utilization.

2.3.1.2 Isolation of *Clostridium Perfringens*

Two samples were screened for *Cl. perfringens*. The presumptive colonies of black color with opaque white zone surrounding the colonies as a result of lecithinase activity on TSC agar were observed. Ten isolates were re-streaked on TSC agar and incubated under anaerobic and standard conditions. All 10 *Cl. perfringens* isolates were able to grow under anaerobic condition only. Five isolates were chosen and identified by biochemical reactions (Table 2.2). All biochemical characteristics of *Cl. perfringens* isolates were similar except for isolate CP3 which was unable to hydrolyze gelatin.

Table 2.2 Biochemical characteristic profiles of *Cl. perfringens* isolated from chicken intestine on TSC agar

Bacterial species	Colony on TSC agar	Motility	Catalase test	Nitrate reduction test	Lactose fermentation	Gelatin liquification
Cl. perfringens isolated from food in Khon Kaen, Thailand	Black	- [a]	-	+ [a]	A/G [a]	+
CP2 [b]	Black	-	-	+	A/G	+
CP3	Black	-	-	+	A/G	-
CP4 - CP6	Black	-	-	+	A/G	+

[a] Reaction symbols, +: positive results; -: negative results; A/G: acid and gas production

[b] Isolated strains of bacteria from chicken intestine, CP, *Cl. perfringens* isolated on TSC agar

2.3.1.3 Isolation of *Escherichia coli*

In this investigation, *E. coli* was isolated from only samples from

farm A and farm D. Thirteen isolates which were able to produce metallic green sheen on EMB agar (E1-E13) were collected. Only 7 isolates of the presumptive (E1-E7) and 5 isolates of the non-presumptive (C2-C6) *E. coli* colonies were randomly chosen and tested for their biochemical properties. Almost all biochemical characteristic properties of *E. coli* isolates were similar to the reference strain (Table 2.3). However, minor variations in the carbohydrate utilization were observed in isolate E3 (no gas production from rhamnose utilization), isolate E4 (negative for rhamnose utilization) and isolate E7 (negative for xylose utilization) (Table 2.3). All 5 isolates of non-*E. coli* bacteria (C2-C6) were Gram-negative rod shape with no metallic green sheen and different colony morphology on EMB and MacConky agar. The biochemical characteristic profiles of IMViC test observed from non-*E. coli* bacteria were different from *E. coli* (Table 2.3). These results confirmed that they were non-*E. coli* bacteria.

2.3.1.4 Isolation of *Listeria monocytogenes*

The isolation of *L. monocytogenes* was performed from all samples. Seventeen presumptive *Listeria* colonies of dark or with dark halo on esculin containing medium (PALCAM) were observed from only one sample. All 17 typical *Listeria*-colonies were identified using Gram staining and biochemical reactions. All isolates were Gram-positive rod shape with biochemical characteristic similar to *L. monocytogenes* and *L. innocua*. Results of only isolates LM1-LM7 are shown in Table 2.4. All isolates were further tested for β-hemolytic reaction using sheep blood agar (OXIOD). The results showed that all isolates were negative for β-hemolytic reactions indicating that they are all *L. innocua*. These investigations demonstrated that there was no *L. monocytogenes* presents in the 5 samples of chicken intestines from 4 different farms in Nakhon Ratchasima. However, dark colonies with dark halo

and other different colony morphologies were also observed on PALCAM agar. Seven isolates of non-*Listeria* bacteria were collected from two samples and 4 of them (L2, L4, L5 and L6) were randomly chosen and identified by Gram staining and biochemical reactions. All non-*Listeria* bacteria were Gram-negative with different biochemical profiles from the genus of *Listeria* (Table 2.4). These results indicated that the condition for enrichment and isolation of *Listeria*-bacteria was also able to enrich other bacteria. One isolate of non-*Listeria* bacteria, L6, should be *Salmonella* sp. because the biochemical profiles were similar to *Salmonella* sp. isolate BC5 (Table 2.5).

2.3.1.5 Isolation of *Salmonella*

The presumptive colonies of *Salmonella* spp. were observed from sample 1 after the enrichment step. Red colonies with black center and black colonies with black halo were found from XLD and BS agar, respectively. Twelve isolates were randomly collected. Five isolates from RV broth (BC1-BC5) and 4 from TT broth (S1-S4) were characterized by biochemical reactions. Almost all biochemical profiles of *Salmonella* sp. isolated strains were similar to the reference strains except for 2 isolates from TT broth (S1 and S2) and 1 isolate from RV broth (BC5) (Table 2.5). The S1 and S2 hydrolyze gelatin slowly. The BC5 was unable to utilize myo-inositol (Table 2.5). These results indicated that minor variation in the biochemical characteristics were found from *Salmonella* sp. isolated from chicken intestines. For non-presumptive colonies, different colony morphologies of 2 isolates from RV broth (RV2 and RV3) and 1 isolate from TT broth (TT1) were observed on XLD and BS agar. Results of biochemical characteristic profile of non-presumptive *Salmonella* colonies confirmed that they were not *Salmonella* sp. (Table 2.5).

Table 2.3 Biochemical characteristic profiles of *E. coli* and non-*E. coli* bacteria isolated from chicken intestine on EMB agar

Bacterial species	Catalase test	Oxidase test	Motility	H_2S production	Urea hydrolysis	Gelatin hydrolysis	Nitrate reduction	Carbohydrate utilization						IMViC			
								Dextrose	Lactose	Manitol	Xylose	Myo-inositol	Rhamnose	Indole production	Methyl red reactive compound test	Voges-proskauer (VP) reaction	Citrate test
E. coli TISTR 887[b]	+ [a]	- [a]	+	-	-	-	+	A/G [a]	A/G	A/G	A/G	-	A/G	+	+	-	-
E1 [c]	+	-	-	-	-	-	+	A/G	A/G	A/G	A/G	-	A/G	+	+	-	-
E2	+	-	-	-	-	-	+	A/G	A/G	A/G	A/G	-	A/G	+	+	-	-
E3	+	-	-	-	-	-	+	A/G	A/G	A/G	A/G	-	A [a]	+	+	-	-
E4	+	-	+	-	-	-	+	A/G	A/G	A/G	A/G	-	-	+	+	-	-
E5	+	-	-	-	-	-	+	A/G	A/G	A/G	A/G	-	A/G	+	+	-	-
E6	+	-	+	-	-	-	+	A/G	A/G	A/G	A/G	-	A/G	+	+	-	-
E7	+	-	+	-	-	-	+	A/G	A/G	A/G	-	-	A/G	+	+	-	-

Table 2.3 (Continued)

Bacterial species	Catalase test	Oxidase test	Motility	H_2S production	Urea hydrolysis	Gelatin hydrolysis	Nitrate reduction	Carbohydrate utilization						IMViC			
								Dextrose	Lactose	Manitol	Xylose	Myo-inositol	Rhamnose	Indole production	Methyl red reactive compound test	Voges-proskauer (VP) reaction	Citrate test
E. coli TISTR 887[b]	+ [a]	- [a]	+	-	-	-	+	A/G [a]	A/G	A/G	A/G	-	A/G	+	+	-	-
C2 [c]	+	-	-	-	-	-	+	ND [a]	A/G	A/G	A/G	ND	ND	-	-	+	+
C3	+	-	+	-	-	-	+	A/G	-	-	A/G	A/G	A/G	-	-	+	+
C4	+	-	-	-	-	-	+	A/G	A	A/G	A/G	A/G	A/G	-	-	+	+
C6	+	-	+	+	+	+	+	A/G	-	-	-	-	A	-	+	-	-

[a] Reaction symbols, +: positive results; -: negative results; A: acid production; A/G: acid and gas production; ND: not determine

[b] Type strains, TISTR: Thailand Institute of Scientific and Technology Research

[c] Isolated strains of bacteria from chicken intestine, E: *E. coli* isolated on EMB agar; C, non-*E. coli* bacteria isolated on EMB agar

Table 2.4 Biochemical characteristic profiles of *Listeria* and non-*Listeria* bacteria isolated from chicken intestine on PALCAM agar

Bacterial species	Catalase test	Oxidase test	Motility (in MTM medium at 25°C)	H_2S production (in SIM medium)	Urea hydrolysis	Gelatin hydrolysis	Nitrate reduction	Carbohydrate utilization							IMViC			
								Dextrose	Lactose	Manitol	Xylose	Myo-inositol	Rhamnose	Moltose	Indole production	Methyl red reactive compound test	Voges-proskauer (VP) reaction	Citrate test
L. monocytogenes DSM 12464[b]	+[a]	-[a]	Umbrella shape	-	-	-	ND[a]	A[a]	ND	-	-	ND	A	A	-	+	+	ND
L. innocua DSM 20649[b]	+	-	Umbrella shape	-	-	-	ND	A	ND	-	-	ND	A	A	-	+	+	ND
Listeria sp. JCM 7679[b]	+	-	Umbrella shape	-	-	-	ND	A	ND	-	A	ND	-	A	-	+	-	ND
LM1-LM7[c]	+	-	Umbrella shape	-	-	-	ND	A	ND	-	-	ND	A	A	-	+	+	ND
L2, L4[c]	+	-	-	-	-	-	+	G[a]	A/G[a]	A/G	A/G	A/G	A/G	A/G	-	-	+	+
L5	+	-	-	-	-	-	+	A/G	-	-	-	A	-	-	+	+	+	+
L6	+	-	+	+	-	-	+	A/G	-	A/G	A/G	-	A/G	A/G	-	+	-	+

[a] Reaction symbols, +: positive results; -: negative results; A: acid production; G: gas production; A/G: acid and gas production; ND: not determine

[b] Type strains, DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH from German Collection of Microorganisms and Cell Cultures; JCM: Japan Collection of Microorganisms

[c] Isolated strains of bacteria from chicken intestine, LM,: *Listeria* sp. isolated on PALCAM agar; L, non-*Listeria* bacteria isolated on PALCAM agar

Table 2.5 Biochemical characteristic profiles of *Salmonella* and non-*Salmonella* bacteria isolated from chicken intestine on XLD and mCCDA agar

Bacterial species	Catalase test	Oxidase test	Motility	H_2S production	Urea hydrolysis	Gelatin hydrolysis	Nitrate reduction	Carbohydrate utilization						IMViC			
								Dextrose	Lactose	Manitol	Xylose	Myo-inositol	Rhamnose	Indole production	Methyl red reactive compound test	Voges-proskauer (VP) reaction	Citrate test
S. Typhimrium TISTR 292 [b] and *S.* Enteritidis JCM 1652 [b]	+[a]	-[a]	+	+	-	-	+	A/G [a]	-	A/G	A/G	A/G	A/G	-	+	-	+
S1[c]	+	-	+	+	-	+	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
S2	+	-	+	+	-	+	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
S3	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
S4	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
BC1 [c]	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
BC2	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
BC3	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
BC4	+	-	+	+	-	-	+	A/G	-	A/G	A/G	A/G	A/G	-	+	-	+
BC5	+	-	+	+	-	-	+	ND [a]	-	A/G	A/G	-	A/G	-	+	-	+

Table 2.5 (Continued)

Bacterial species	Catalase test	Oxidase test	Motility	H_2S production	Urea hydrolysis	Gelatin hydrolysis	Nitrate reduction	Carbohydrate utilization						IMViC			
								Dextrose	Lactose	Manitol	Xylose	Myo-inositol	Rhamnose	Indole production	Methyl red reactive compound test	Voges-proskauer (VP) reaction	Citrate test
S. Enteritidis JCM 1652 [b]	+[a]	-[a]	+	+	-	-	+	A/G [a]	-	A/G	A/G	A/G	A/G	-	+	-	+
CM2 [c]	+	-	+	+	-	-	+	ND	-	A/G	A/G	A [a]	A/G	-	+	-	+
CM7	+	-	+	+	-	-	+	ND	-	A/G	A/G	A	A/G	-	+	-	+
RV2 [c]	+	-	-	-	-	-	+	A/G	A/G	A/G	A/G	A/G	A/G	-	-	+	+
RV3	+	-	+	-	-	-	+	A/G	-	A/G	A/G	-	A/G	+	+	-	-
TT1 [c]	+	-	+	+	+	+	+	A/G	-	-	-	-	A	-	+	-	-

[a] Reaction symbols, +: positive results; -: negative results; A: acid production; A/G: acid and gas production; ND: not determine

[b] Type strains, TISTR: Thailand Institute of Scientific and Technology Research; JCM: Japan Collection of Microorganisms

[c] Isolated strains of bacteria from chicken intestine, BC, *Salmonella* sp. enriched using RV broth and isolated on XLD agar; CM, *Salmonella* sp. isolated on mCCDA agar; RV, non-*Salmonella* bacteria enriched using RV broth and isolated on XLD agar; S: *Salmonella* sp. enriched using TT broth and isolated on XLD agar; TT, non-*Salmonella* bacteria enriched using TT broth and isolated on XLD agar

2.4 Discussions

Isolation and characterization of foodborne pathogens and food safety indicators include *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp., *Clostridium perfringens*, and *Listeria monocytogenes* from 5 fresh chicken intestines of 4 different farms in Nakhon Ratchasima, Thailand were attempted. Only *E. coli*, *Salmonella* spp. and *Cl. perfringens* were found in this investigation.

For the prevalence of *Campylobacter* spp. and *L. monocytogenes* in Thailand, especially from chicken intestine has yet to be report. In this study, contamination of *L. monocytogenes* were not found from chicken intestines. These results agree with the investigation of Minami and colleagues (2010) who reported that *L. monocytogenes* in chicken meat from Bangkok and Pathum Thani, Thailand were very low in supermarket (4%) and open market (6%) samples (Minami et al., 2010). In meat products, only 23.3% and 10% of pork intestine and chicken liver were contaminated with *L. monocytogenes*, respectively (Stonsaovapak and Boonyaratanakornkit, 2010). The results of their study indicated that incident of *L. monocytogenes* contaminated in meat and meat products were very low. The sources of contamination of *Listeria* spp. in frozen, ready-to-eat, roasted, steamed, and fried chicken meat products from a plant in Thailand was the equipment surfaces that direct contact with the products (Lekroengsin et al., 2007). These results indicated that the contamination of this bacterium should be the environmental sanitation problem. No *L. monocytogenes* was found in chicken intestine in our research. *Camp. jejuni* and *Camp. coli* are the most common pathogen from poultry (Lilja and Hänninen, 2001).

However, no contaminations of *Campylobacter* in chicken intestine were found in our investigation. The prevalence of *Campylobacter* from different sampling

parts has been reported in many countries including Thailand. *Campylobacter* was isolated from 14.4% of intestinal of live birds but was not found from chicken carcasses in Accra Metropolitan (Sakey et al., 2001), from 49.9% chicken meat in Ireland (Whyte et al., 2004), from 70.6 % chicken feces from 34 different farms in Northern part of Spain (Esteban et al., 2008). In Thailand, prevalence of *Campylobacter* in chickens from farms, slaughterhouses, and markets ranging from 38- 64% in Chiang Mai and Lamphang provinces of northern Thailand (Padungtod and Kaneene, 2005) and 24- 28% in chicken meat from market in Bangkok (Vindigni et al., 2007), and 11% in chicken gizzard, 1% in chicken breast meat from retail sale outlets in Khon Kaen Province (Noppon et al. 2008). All information reported earlier indicated that poultry is the common source of the *Campylobacter* contamination. Moreover, the contamination level of pathogens from chicken in different local area and sampling parts were also different. Different parts of an animal sampling may possess varying levels of *Campylobacter* contamination. Samples containing carcass rinse fluid and neck-skin detected higher *Campylobacter* count in more chickens than examination of the neck-skin sample alone (Jørgensen et al., 2002). Therefore, the contradicting results of low prevalence of *Campylobacter* in poultry observed in this investigation might be from the effects of several factors such as different sampling parts of chicken were tested or chicken intestine samples were collected from different local area. However, more samples should be investigated from different farms to confirm the low prevalence of these pathogens from chicken intestine in Nakhon Rathasima.

Prevalence of *Salmonella* spp. from several sources in Thailand were investigated in human (Bangtrakulnonth et al., 2004), pork, chicken meat and human in

Khon Kaen (Angkititrakul et al., 2005), raw beef, chicken, pork, and chicken eggs in Bangkok (Vindigni et al. 2007; Minami et al. 2010), food animal and food for human in northern Thailand (Padungtod and Kaneene, 2005). However, information of the isolation and characterization of *Salmonella*, *E. coli* and *Cl. perfringens*, from chicken intestines in Thailand are still lacking. Therefore, the isolation and characterization of these bacteria were performed. Some minor different biochemical characteristic profiles were found from each bacterial isolate especially carbohydrate utilization and gelatin hydrolysis. These results indicated that the diversity of phenotypic intestinal bacteria was observed. Interesting, *Salmonella* sp. could be found from the enrichment culture of *Campylobacter* spp. and *L. monocytogenes* with showed some minor variation in carbohydrate utilization characteristics when compared to the isolates obtained from RV and TT broth (Table 2.4 and 2.5). Moreover, the biochemical profiles of isolate RV3 (Table 2.5) which was non-*Salmonella* bacteria isolated from enrichment culture of *Salmonella* was similar to *E. coli* excepted that this isolate was unable to utilize lactose. These results indicated that identification of closely related bacteria using only biochemical profiles is difficult. Processing of large numbers of samples is not easy in general, 10 or more tests may be necessary for differentiation of the species within a group (Settanni and Corsetti, 2007). Therefore, molecular-based methods were developed as a more rapid method for pathogenic detection.

The specificity of detection method by molecular-based technique depends on the target gene of interest and the bacterial background from the sample. As shown in this investigation, some *Salmonella* sp. and other non-target bacteria were able to grow in specific cultivation conditions of *Listeria* spp. and *Campylobacter* spp.

Furthermore, the investigations of specificity of target genes in isolated strains in Thailand are still limited. Therefore, the isolated bacterial strains including target and non-target bacteria obtained from this part were used as tested organisms to evaluate the specificity of the developed multiple target bacteria detection methods from the enrichment culture in Chapter 3 and 4.

2.5 Conclusions

The investigation of foodborne pathogens from 5 chicken intestines of 4 commercial farms showed that no *L. monocytogenes* or *Campylobacter* spp. were detected. Characterization of *Salmonella* sp., *E. coli*, and *Cl. perfringens* were done and found that only minor different biochemical characteristics were found from each isolate. However, more investigation and characterization of intestinal bacteria from chicken of different farms are needed to evaluate trends in the occurrence of these pathogens and to measure the efficiency of farms managements. So that, the foodborne pathogen contamination can be reduced by elimination or minimization step of pathogens carriage at the food chain production. Lastly, the bacteria isolated in this part were used as the tested organisms for rapid methods development to increase the specificity of the foodborne pathogens detection methods.

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CHAPTER III

NOVEL 16S rDNA BASED OLIGONUCLEOTIDE ARRAY TO SPECIFICALLY DETECT FOODBORNE PATHOGENS

Abstract

Oligonucleotide array hybridization based methods can be used as a method for screening of multiple foodborne pathogens. Several target pathogens can be monitored in a single step of DNA hybridization using suitable specific probes on an array matrix. In this investigation, screenings of suitable probes for specific detection of foodborne pathogens prevalence in fresh chicken meat were performed using post-PCR labeled target regions. The hybridization signals of non-radioactive labeling digoxigenin (DIG) incorporated purified PCR target regions were observed by naked eyes. The target regions of 16S rRNA gene specific for *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens*, and *prf*A gene specific for *L. monocytogenes* were used as models. The optimum concentration of the oligonucleotide probes was found to be 200 pmol. The labeled target regions of 16S rRNA and *prf*A genes generated by post-PCR labeling methods were successfully used for the differentiation of target bacteria in both the genus and species levels, respectively. Detection of multiple target bacteria by oligonucleotide array hybridization targeted to the 16S rRNA genes showed that large number of

target bacteria can be distinguished at the genus level with easy result interpretation. The detection systems in this investigation were carried out to detect multiple target bacteria at as low as 1 ng in the mixed DNA of the 5 bacterial species. However, some cross-reactivities were found from non-target bacteria isolated from the enrichment culture.

3.1 Introduction and review literature of foodborne pathogen detection method

The development of novel alternatives for the monitoring, characterization and identification of foodborne pathogens is a key process in food industry (Rodríguez-La´zaro et al., 2007). Foodborne pathogen detection methods which have been applied in poultry meat are discussed in this part.

3.1.1 Conventional methods

The culturing and plating method is the oldest bacterial detection technique and remains the standard detection method. Classical cultural methods including step of pre-enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification have been applied to detect foodborne pathogens (Boera and Beumer, 1999; Lazcka et al. 2007). Several methods have been developed for samples collection for bacteriological examination from poultry carcasses. Generally, the swab technique and the rinse method have been applied. However, there are some variation in using these methods depending on the worker (Mountney and Parkhurst, 1995). Standard culture methods for detecting *Salmonella* spp. and *Campylobacter* spp. in poultry involve whole carcass rinses, and enrichment in selective agar. The completion time

for these culture assays is typically 48-96 h and sensitivity of detection was > 10 CFU/ml (Mandrel and Wachtelt, 1999).

The limitation of this method is that the whole procedure is time consuming which depend on the enrichment and selective culture, the biochemical analysis for bacteria of interest. In the case of *Campylobacter*, 4–9 days are needed to obtain a negative result and between 14 and 16 days for confirmation of a positive result. Different selective media are used to detect particular bacteria species. They can contain inhibitors (in order to stop or delay the growth of non-target strains) or particular substrates that only the target bacteria can degrade or that confers a particular color to the growing colonies. Detection is then carried out using optical methods, mainly by ocular inspection (Lazcka et al., 2007). Furthermore, processing of large numbers of samples is not easy in general, 10 or more tests may be necessary for differentiation of the species within a group. These indicated that cultivable methods are labor intensive, time consuming, and not always reliable. In the food industry, rapid methods to provide high accuracy of the possible presence of pathogens in raw materials and finished food products are needed (Boera and Beumer, 1999; Settanni and Corsetti, 2007; Moa et al., 2008).

3.1.2 Rapids methods

The efficiency of novel rapid methods for pathogen detection have been focused on increase sensitivity, reduce time-consumption and can be used as high-throughput detection method.

3.1.2.1 Immunological based methods

Immunological methods rely on the specific binding of an antibody to an antigen. In case of immunomagnetic separation (IMS), a pre-treatment

and/or pre-concentration step, can be used to capture and extract the target pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it. Alternatively, the enzyme-linked immunosorbent assay (ELISA) test is the most established technique. ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. Schematic representation of the sandwich-ELISA protocol is shown in Figure 3.1.

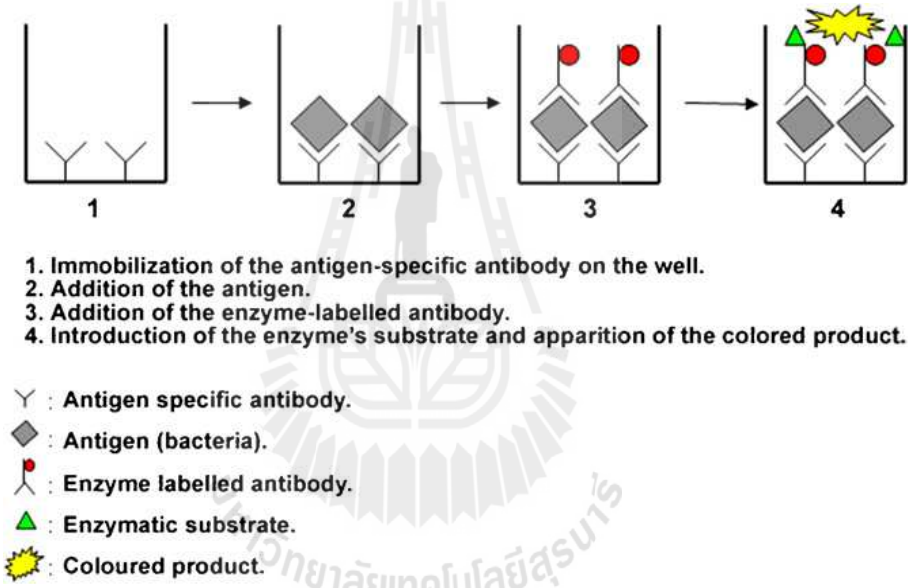

Figure 3.1 Schematic representation of the sandwich-ELISA protocol (Lazcka et al., 2007)

The suitability of these antibodies depends mainly on their specificity. Polyclonal antisera contain an assortment of antibodies having different cellular origins and, therefore, somewhat different specificities. One of the disadvantages of using polyclonal antisera in immunological assay is the variability found in animal's immune response. Therefore, the developments of monoclonal

antibodies greatly enhance the field of immunological assay by providing a consistent and reliable source of characterized antibodies (Boer and Beumer, 1999). The application of immunological based method for foodborne pathogens detection in previous research was described below.

In 2001, Lilja and Hänninen detected a thermophilic *Campylobacter* spp. from poultry product samples using Enzyme-Linked-Immunosorbent-Assay (ELISA) methods. After 46-50 h of enrichment, *Campylobacter* spp. detection using ELISA based method were performed. ELISA analysis is based on the sandwich-technique, which used two different polyclonal antibodies against the *Campylobacter* spp. Their results showed that ELISA method was able to detect the thermophilic *Campylobacter* spp. and revealed no other species. The entire procedure starting from enrichment to final results took only 2.5 working days. However, two samples showed ELISA positive results while negative in culture and PCR methods which may indicate false-positive or they might have some other *Campylobacter* spp. (Lilja and Hänninen, 2001) in the samples. In 2003, Hong and colleagues investigated the detection of *Camp. coli*, *Camp. jejuni*, and *S. enterica* on poultry carcasses by combination of PCR and ELISA. PCR-ELISA involves incorporation of chemically tagged nucleotides into the PCR amplicon. After amplification, PCR products can be detected with antibody-enzyme conjugate that recognizes the unique chemical label presenting in the incorporated PCR product. PCR were performed after increasing of target bacterial pathogen in an enrichment step. Primers and probes were designed based on the *Salmonella* invasion gene (*inv*A) and the *Campylobacter ceu*E gene, which encodes a lipoprotein involved in siderophore transport. A biotin molecule was added to the 3’ end of the probe to

prevent it from serving as a primer in the PCR, and this oligonucleotide was used to bind the PCR amplicon to the bottom of the ELISA plate coated with streptavidin. PCR products which were labeled with digoxigenin (DIG) during amplification can be detected by anti-DIG antibody–peroxidase conjugate. Their results showed that the detection limit of 40 PCR cycles PCR-ELISA of *Campylobacter* sp. was as low as 346 fg which is equivalent of 40 CFU/ml and 2×10^2 CFU/ml of *S. enterica*. However, 5% false positive (positive for PCR-ELISA but negative for cultural method) and 8.3% false negative (negative for PCR-ELISA but positive for cultural method) were seen when *Salmonella* was detected directly (without enrichment) from chicken carcass rinse and 6.6% false positive, 0.16% false negative from samples following overnight enrichment culture (Hong et al., 2003). This indicated that the accuracy of this technique depend on the detection limit which is related to step of enrichment.

In 2005, Bohaychuk and colleagues evaluated three rapid technologies include ELISA, PCR, and Lateral Flow Immunoprecipitation for the detection of *Salmonella* Newport AMP^R, *Camp. jejuni*, *L. monocytogenes*, and *E. coli* O157:H7 in meat and poultry products. The enrichment steps were performed prior to pathogen detection. They reported that using commercial ELISA kit (TECRA *Salmonella* Visual Immunoassay, International Bioproducts Inc., Vaughn, Ontario, Canada), they were able to detect *Salmonella* at the same number of positive and negative samples as the culture method. However ELISA test (TECRA *Campylobacter* Visual Immunoassay, TECRA International Pty Ltd., Willoughby, New South Wales, Australia) gave more positive results than the culture method for the detection of *Camp. jejuni* in chicken leg sample including positive for

uninoculated chicken leg. Moreover, the researchers reported that the ELISA kit (*Listeria*-Tek, Organon Teknika Corporation, Durham, N.C.) assay did not detect the same number of positive samples as culture for the detection of *L. monocytogenes* in chicken leg product. Using of ELISA test for *Listeria* spp. the detection had a very low sensitivity rate of 62% compared with the culture method. These results indicated that the ELISA assay, as used in their study, did not perform well in detecting *Campylobacter* spp. and *Listeria* spp. The researcher discussed that the higher number of positive results might be due to the presence of naturally occurring microflora on the chicken leg product. Thus it is probable that there were nonspecific reactions of the ELISA assay in this sample (Bohaychuk et al., 2005).

Antibody based method on the detection of surface antigens has weak point in term of cross-reaction. Because of the low accuracy results, antibody method is not suitable for pathogen detection (Kim et al., 2007).

3.1.2.2 PCR based methods

Polymerase chain reaction (PCR) has become the most frequently used method for amplifying nucleic acids of interest. It is based on the isolation, amplification and quantification of a short DNA sequence including the target bacteria's genetic material. The reaction system includes a heat-stable DNA polymerase, a template DNA from the pathogens being detected, and two complementary oligonucleotide primers that are designed to flank the sequence on the template DNA. A typical amplification needs 20 to 40 cycles, which amplifies specific pieces of template DNA at more than a billion-fold. The presence of the amplified sequence is subsequently detected by gel electrophoresis. It is possible to

significantly reduce assay times by PCR based methods while maintaining a high level of sensitivity and specificity. These methods are also able to distinguish closely related species which most antibody tests could not. Different PCR methods have been developed for bacterial detection including real-time PCR, multiplex PCR, and reverse transcriptase PCR (Lazcka et al., 2007; Nugen and Baeumner; 2008; Shi et al., 2010).

Application of PCR based methods for foodborne pathogens detection in poultry and chicken meat has been reported in many research. In 2002, Whyte and colleagues investigated the prevalence of *Salmonella* contamination in raw poultry using PCR technique. Primers were designed to amplify fragments within a 1.8 kb *Hin*dIII DNA sequence. This amplified region is specific to a wide range of *Salmonella* serotypes. They showed that level of detection of the PCR test using pure cultures of *S.* Kentucky was 10 CFU. For non-sterile skin samples spiked with a nalidixic acid/streptomycin resistant strain of *S.* Enteritidis, the detection limit was 10^4 CFU. However they claimed that the sensitivity can be increased approximately 100-fold by using nested PCR amplification reaction. The researchers suggested that DNA based techniques are rapid and more sensitive than the traditional culture method for the detection of *Salmonella* in raw poultry (Whyte et al., 2002). In 2005, Nierop and colleagues investigated the contamination of *Salmonella*, *L. monocytogenes*, and *Campylobacter* in chicken carcasses (Gauteng; South Africa) using conventional culture and PCR based methods. For PCR technique, bacterial DNA was extracted from cultural broth specific for each pathogen. Set of primers were designed to amplify gene specific for *Salmonella* (*inv*A gene), *Camplylobacter*, and *L. monocytogenes* (*hly*A gene). Real-time PCR technique was applied to detect

Salmonella and *Campylobacter*. For detection of *L. monocytogenes*, standard PCR were applied. The detection limit of DNA based method was 200 CFU/ml for both *Salmonella* and *Campylobacter*, and 150 CFU/ml for *L. monocytogenes*. The researchers also reported that more samples were found containing each pathogen by PCR analysis than by cultural method (Nierop et al., 2005).

In 2006, Neubauer and Hess developed a multiplex PCR suitable for differentiating foodborne pathogens belonging to genera *Campylobacter*, *Helicobacter* and *Arcobacter* which can be isolated from poultry and humans. Primers were designed based on variable regions of 16S rRNA gene specific for each bacterial genus. One common reverse primer and three genus-specific forward primers were applied in a single step PCR procedure. Their results showed that parts of the 16S rRNA gene of all species tested (*Campylobacter*, *Arcobacter* and *Helicobacter*) can be amplified by the developed primers in their research. But no amplified product were obtained from the non-thermophilic *Campylobacter*, *Camp. hyointestinalis* and *Camp. fetus* (Neubauer and Hess, 2006). However, no detection limit or sensitivity of this technique was reported.

In 2006, Cortez and colleagues identified *Salmonella* spp. isolated from chicken abattoirs by multiplex PCR method. Primers were targeted to amplify the genes *inv*A (specific for genus *Salmonella*), *sef*A (fimbrial antigen of *S.* Enteritidis) and *pef*A (plasmid-encoded fimbria of *S.* Typhimurium). After *Salmonella* from various collected samples were isolated, bacterial DNA extraction were performed and used as template for multiplex PCR analysis. They suggested that the *pef*A gene can be amplified both from *S.* Enteritidis and *S.* Typhimurium. However the differentiation can be done by the presence of a *Kpn*I restriction site in

S. Typhimurium amplicon in which it does not exist in *S*. Enteritidis amplicon (Cortez et al., 2006). However no detection limit or sensitivity of this technique was reported.

For available commercial kit, TaqMan pathogen detection kit has been generated for detection of pathogens including *S. enterica*, *E. coli* O157:H7, *L. monocytogenes*, and *Camp. jejuni*. The detection method was based on real-time PCR using extracted bacterial DNA from pre-enriched culture. In *S. enterica*, after incubation for 16 h, sensitivity of detection from all spiked food samples (beef 7% fat, beef 20% fat, eggs, and chocolate) is 1 CFU in 25 g of these food sample and 6 cells in 5 g of spiked chicken. Detection of *Camp. jejuni* and *L. monocytogenes* from spiked chicken showed sensitivity of <1 CFU/25 g sample using real-time PCR after step of enrichment (TaqMan® Pathogen Detection Kits; Applied Biosystem, USA). Alternatively, The BAX® System for detection of *Camp. jejuni*, *Camp. coli*, *Camp. lari*, *L. monocytogenes* and *Salmonella* has been applied. Detection system performs by combining all PCR reagents into a single tablet. This tablet conveniently packaged inside the PCR tubes contained in each kit. For *Camp. jejuni*, *Camp. coli*, *Camp. lari* detection system is based on real-time PCR. Contaminated samples can be enriched for 24-48 h before processing with sensitivity as low as 10^4 CFU/ml in poultry carcass rinses. For *L. monocytogenes* and *Salmonella*, after standard enrichment, bacteria in samples were lysed to break open the cell walls and released DNA. Processing in automated unit using PCR based method takes less than 4 h with sensitivity/specificity rates of 98% (BAX® system; DuPont Qualicon, Singapore).

In conclusion, the specificity of PCR-based method depends on target gene and detection limit which related to step of enrichment. When amplified product from different bacterial species have the same size, they cannot be used to

differentiate the bacterial species, another identification steps are required. For available commercial kit, both systems show high sensitivity for bacterial detection using real-time PCR-based methods. However, these systems require specific compatible of all chemical reagents, complicated equipment for signal detection and data evaluation. One of the major drawbacks of the PCR methods is that the number of species that can be analyzed in each reaction is limited. Even though multiplex PCR is able to amplify multiple targets by including several sets of target-specific or degenerated primers in a single tube, the detection capability is still restricted to a few targets per assay because of the low resolution of agarose gels in traditional PCR or the limited choices of fluorescent detectors in real-time PCR (Severgnini et al., 2011). Thus PCR-based methods are suitable for pathogen detection in term of rapid method but the step of PCR product validation, with easy, low cost but high sensitivity, should be improved.

3.1.2.3 Oligonucleotide array based method for multiple bacteria detection

Oligonucleotide array hybridization is interesting for monitoring foodborne pathogen. Microarray technology is a powerful tool that can be used for simultaneous detection of thousands of genes or target DNA sequences (Wang et al., 2002). An essential feature of the DNA array technique is the hybridization of labeled DNA with arrays of immobilized probes (Gauthier and Blais, 2003). Nucleic acid hybridization occurs between DNA or RNA from target organisms and a DNA probe (~15-30 nucleotides) which has sequence complementary to the target sequences (Boera and Beumer, 1999). Normally oligonucleotide probe sets spotted onto nylon membranes have been used for the diagnosis of bacteria in various environmental

systems (Bodrossy and Sessitsch, 2004). The target molecule to be analyzed, such as DNA, is labeled and hybridized to the recognition probes on the array. The signal generated by the bound labeled target on the array allows identification based on the known locations of the probes. The main steps in the design and implementation of a DNA microarray experiment are summarized here i) probe development; ii) array fabrication; iii) sample preparation; iv) hybridization assay; v) detection; and vi) data analysis (Rasooly and Herold, 2008). This method can be applied for multiple pathogens detection in a single step. Microarray microbial genotyping analysis is shown in Figure 3.2.

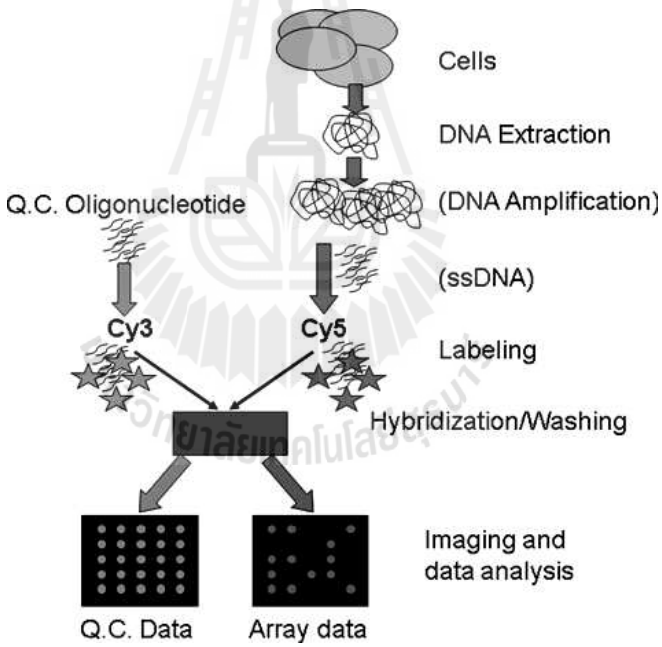

Figure 3.2 Microarray microbial genotyping analysis. Genomic target DNA is extracted from a cell, amplified, and converted to single-stranded DNA (if needed). The DNA can be labeled, during or after amplification, with a fluorescent dye (e.g., Cy5). In some applications, the labeled target DNA is mixed with a quality control (QC) oligonucleotide (complementary to a QC oligonucleotide printed in each spot) which is labeled with a different fluorescent dye. A QC scan, with signals at every spot on the array, can be used to verify proper printing and hybridization of the microarray (Rasooly and Herold, 2008).

Probe selection and design is an important first step in microarray-based pathogen detection because it can impact the overall fidelity of the assay especially regards to the levels of specificity and sensitivity (Uttamchandani et al., 2008). Moreover the sensitivity and specificity of oligonucleotide-based arrays also depend on several factors such as genetic dissimilarity, position of mismatch, and secondary structure of targets (Eom et al., 2007).

For pathogen detection using microarrays method, various target genes have been used for identifying pathogens such as 16S rDNA, 23S rDNA, 16S-23S rDNA internal transcribed spacer region (ITS), and other genes that code for β-galactosidase, elongation factors Tu, F1F0 ATPase, RecA protein, and Hsp60 heat shock protein, *rpo*B gene (Yoo et al., 2004). The target genes may be species-specific such as pathogenic or virulence genes that can be easily identified by a simple PCR. Moreover, a complex multiplex PCR with a mixture of many primers can be used to amplify many target genes specific for each contaminated bacteria. However, using different primers for different species is impractical in oligonucleotide array technology, especially in the case of a specimen or unknown sample containing one or more possible bacteria. Thus consensus genes among many pathogenic bacteria which can be amplified by a single pair of universal primers are more suitable. This amplified products containing variable regions specific for bacterial species are then distinguished from each other by hybridization with specific oligonucleotide probes (Wang et al., 2007). In 2011, Hu and colleagues reported the application of oligonucleotide array using the heat shock protein gene (*gro*EL) as target for multiple foodborne pathogen detection. Digoxigenin-linked enzyme color development method with the results evaluated by naked eye was used for detection of hybridization signal.

Different dilutions of pure culture of *E. coli*, *S. enterica*, *L. monocytogenes*, *Camp. jejuni*, *Vibrio parahaemolyticus* were separately used for detection limit assay. The detection limit of their methods was 10^2 CFU/ml of diluted pure culture. However, cross-reactions were between *E. coli* and *Shigella* spp. reported in their works.

Another consensus gene used as target for multiple foodborne pathogen detection by oligonucleotide array methods is rRNA. Because of the high copy of rDNAs in bacterial genomes, rDNA sequences are commonly used in microbial system (Bavykin et al., 2008). In 2004, Hong and colleagues discriminated 14 species of bacteria causing foodborne infections and two unrelated bacterial species using a mutation region of the 23S rDNA as target probe. In their research, they amplified 23S rDNA by a pair of universal primers and hybridized with synthesized oligonucleotide probes from 21 species-specific which were spotted on the nylon membrane. Digoxigenin-linked enzyme used for detection positive signal from hybridization. Their results showed that only 9 species of pathogenic bacteria performed high sensitivity and specificity for the oligonucleotide array. However, they found that *Salmonella* spp. cross-reacted with *E. coli* when applied this technique with mock samples and true samples. Thus they only concluded that their samples contain *E. coli* or *Salmonella* spp. They also discussed that the high sequence homology or unavailable of 23S rDNA gene database in different species of their genera thus hybridization signals of some species (*Clostridium perfringens* and *Streptococcus pyogenes*) could not be separated. Detection limit of their methods was 10^2 CFU/ml of mixed cells titer from dilution of *E. coli* and *Shigella dysenteriae* and 10^2 -10^3 CFU/ml of mixed cells titer of dilution of *P. vugalis*, *B. cereus*, and *V. cholera* (Hong et al., 2004). The 23S rDNA contains more nucleotides when

compared to 16S rDNA but there are not many 23S rDNA sequences in the databases. Also, by the use of longer probes, it is consequently not helpful for fast and unambiguous identification of closely related species in the DNA array hybridization reactions (Chiang et al., 2006).

The 16S rDNA sequences have been widely selected as the target probe for bacterium detection. There are conserved and variable regions, thus this allows for the design of universal PCR primers and specific hybridization probes. Moreover, there is a large amount of sequence information readily available for many bacteria (Lee et al., 2008). Copies of 16S rDNA in prokaryotic microorganism genome have been published in the ribosomal RNA operon copy number database. The copy number of 16S rDNA in *Camp. coli* and *Camp. jejuni* is 3, *S.* Typhimurium and *E. coli* is 7, and *L. monocytogenes* is 6 (Klappenbach et al., 2001). Using 16S rDNA as the target oligonucleotide probe in various applications of microorganism detections have been reported in a large number of research articles. In 2002, Wang and colleagues developed a microarray method for the detection of predominated 40 bacterial species in the human gastrointestinal tract. They used the sequences of the 16S rDNAs to design 120 oligonucleotide probes that are species-specific of the predominant bacterial species from the human intestine. They demonstrated that all 40 bacterial reference species gave positive results. They also mentioned that 33 species were found in majority of fecal samples. In 2005, Franke-Whittle and colleagues designed a microarray consist of oligonucleotide probes targeting variable regions of the 16S rDNA of plant, animal and human pathogens to investigate microbial communities in the composting process. By microarray strategies, *Streptococcus*, *Acinetobacter lwoffii*, and *Clostridium tetani* in various compost samples were

detected. However they also reported that any organism present at a level of <5% of the total population might not be detected. Thus the ability to detect small population of microorganism in compost may be limited. In 2006, Chiang and colleagues identified *Bacillus* spp., *E. coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. using oligonucleotide array hybridization with 16S rDNA as a target probe. Biotin was used for labeling of the DNA target regions. Their results made clear that the discrimination of these bacterial strains using oligonucleotide array were mainly on the genus level. They suggested that adding more oligonucleotide probes to the array may be required to allow the discrimination of the bacterial strains with low cross-reaction. The detection limit for *E. coli* and *V. parahaemolyticus* was the lowest (5×10^2 CFU/ml of pure culture). In case of *Staphylococcus* spp. and *Bacillus* spp., detection limits was about 4.5×10^3 CFU/ ml.

In 2007, Eom and colleagues applied the oligonucleotide hybridization technique to detect seven selected foodborne pathogen include *Sh. dysenteriae*, *S.* Typhimurium, *S.* Enteritidis, *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *E. coli*, and *Camp. jejuni*. They designed specific oligonucleotide probes based on 16S rDNA information and optimized the hybridization conditions using directly synthesized targets as the model. The optimum condition obtained from the model system was applied to real amplified target for validation. They showed that 60^oC was the optimum hybridization temperature for detection of multiple pathogens when the synthesize oligonucleotide were applied as the target. Using this model system, they successfully detected *Salmonella*, *Vibrio*, and *Campylobacter* species. They found that discrimination was not possible in the cases of different species with high similarity (99% similarity) of the 16S rDNA sequence such as

E. coli and *Sh. dysenteriae* in their model system. The detection limit for their technique was about 1-10 pg of extracted chromosome or about 10^3-10^4 cells.

In 2007, Wang and colleagues developed an oligonucleotide microarray and applied this system for detection of foodborne bacterial pathogens. The researcher used universal primers to amplify a variable region of bacterial 16S rRNA gene. Specific genes including *vir*A and *inv*A were also used for differentiation between *Shigella* and *Salmonella* spp., respectively. The amplified products were then hybridized to species-specific oligonucleotide probes on a glass slide chip. Fluorescence was used for detection of the signal hybridization. Their results found that strains belonging to *Shigella*, *E. coli*, and *Salmonella* are identified only as a class of pathogenic bacteria. They applied this method to identify bacteria isolated from foods. The results indicated that 112 (from total 115) isolated bacteria can be correctly identified (97.4% accuracy). They reported that 10^2 CFU of *E. coli* O157:H7 can be directly detected (no enrichment step) from 1 g of ground beef, fish, and egg samples.

In 2008, Mao and colleagues developed microarray based methods for *Salmonella* spp., *Shigella* spp., *E. coli* detection in clinical samples. Fluorescence was used for signal detection. Target genes for specific detection were 16S rRNA and species or genus specific including *inv*A gene for *Salmonella* detection and *vir*A gene for *Shigella* detection. Their results showed that the probes of the assay were successful in discriminating 14 genera or species of intestinal pathogens. The limit of detection was approximately 10^3 CFU/ml for one species of pathogen.

All available data as reviewed above could be concluded that several research have reported about the specific probes for different bacterial target

groups, but most did not investigate the cross reactivity to the non-target bacteria which are frequently found in enrichment cultures samples. The specificity of detection by these techniques depends on the target gene, target bacteria and non-target bacterial background in the sample. Thus probes specific for the selected bacteria in this investigation were developed and tested for their specificity with non-target bacteria to avoid cross-reaction of the assay.

For bacterial identification by oligonucleotide array based system, the probe selection step is very important. Suitable probes with high signal intensity with the specific target and no-cross reactivity with the non-target bacteria are the key success for this system. To test probe specificity, the system that generates strong hybridization signal should be applied to confirm that no cross-reactivity with other non-target bacteria can be seen. The intensity of signal detection for oligonucleotide array hybridization depends on various parameters. The step of labeling the target is another important factor that affects the hybridization signal. Thus, the suitable DNA labeling process for the target product preparation should be considered for probes selection step. For the DNA labeling process, post-PCR labeling process using fluorescence molecule was found to have the highest signals (Franke-Whittle et al., 2006). Thus post-PCR labeling were applied for preparation of labeled target regions in this research.

Although high-throughput microarray-based techniques have several advantages, but the regular microarray methods need expensive equipments for array development, array scanning and data collection (Bai et al., 2010), which is beyond the budget of many laboratories especially in developing countries. Thus the development of signal investigation system with easy, low cost but high sensitivity

has been required. Easy systems for hybridization signal detection and result interpretation from oligonucleotide array using immunological chromogenic reaction which can be observed by naked eyes have been developed.

The advantage of oligonucleotide array is that the PCR product validation step can be performed in a single step of hybridization with species-specific probes. Thus high-throughput of pathogenic bacteria screening can be performed in short time. Moreover, sensitivity of hybridization signal detection is higher than that of PCR product detection on agarose gel. Therefore the combination of the PCR and oligonucleotide hybridization techniques was used for foodborne pathogen detection in poultry from Thailand. The oligonucleotide probes for hybridization in this part, 16S rDNA were selected as the target regions for probe design since multiple pathogens could be amplified by only a single pair of primer. Moreover, *prf*A gene specific for *L. monocytogenes* detection were also used as a model for specific gene detection by this methods. Thus bacterial pathogen identification using combination of DNA hybridization pattern of 16S rDNA and some species or genus specific genes were performed to improve the accuracy of the detection in the genus and species level.

3.1.3 Objective

Suitable probes were identified for specific detection of foodborne pathogens prevalence in chicken meat. The regulated foodborne pathogen in chicken meat including *E. coli*, *Cl. perfringens*, *L. monocytogenes*, *Staph. aureus* and *Salmonella* spp. were used as models for multiple pathogen detection by oligonucleotide array hybridization using post-PCR labeling process to prepare the

target labeled products. In this experiment, target regions of 16S rRNA and *prf*A gene for *L. monocytogenes* detection were used as models for bacterial detection. Specificity of the probes were tested with several reference and isolated strains of target and non-target bacteria obtained from Chapter 2. Simple systems for hybridization signal detection using immunological chromogenic reaction which can be observed by naked eyes were performed. The hybridization signal pattern of each target bacteria and suitable probes were investigated.

3.2 Materials and methods

3.2.1 Bacterial strains and cultivation

To test for the efficiency and specificity of the probes designed, foodborne pathogens reference and isolated strains from Chapter 2 were selected and tests in this part. Bacterial strains including *E. coli*, *Salmonella* spp., *Staph. aureus* TISTR 517, *Listeria* spp. (*Listeria* sp. JCM 7679, *L. monocytogenes* DSM 12464, *L. innocua* DSM 20649), *Cl. perfringens*, *Shigella* sp. were chosen. All bacterial strain tested in this part are summarized in Table 3.1.

Table 3.1 Bacterial strains used for the validation of 16S rDNA and *prf*A oligonucleotide array probes

Species	**Number of strains**	**Strain number and sources**
Escherichia coli	4	*E. coli* TISTR[a] 887, *E. coli* E[b] 3, 6, 7
Clostridium perfringens	4	*Cl. perfringens* CP[c]1, CP[b]2, 3, 5,
Listeria spp.	3	*Listeria* sp. JCM[a] 7679, *L. innocua* DSM[a] 20649, *L. monocytogenes* DSM 12464

Table 3.1 (Continued)

Species	**Number of strains**	**Strain number and sources**
Salmonella spp.	9	*S.* Enteritidis JCM 1652, TISTR 2394, *S.* Typhimurium TISTR 292, *Salmonella* sp. S[b]2, 3, BC[b] 1, 5, CM[b] 2, L[b]6
Shigella spp.	1	*Shigella* sp. Sh[c]1
Staphylococcus aureus	1	*Staph. aureus* TISTR 517
Non-target bacteria found in enrichment culture	9	C[b]2, 3, 4, 6, RV[b]2, 3, TT[b]1, L[b]2, 5

[a] Reference strains: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH German Collection of Microorganisms and Cell Cultures; JCM, Japan Collection of Microorganisms; TISTR ,Thailand Institute of Scientific and Technology Research

[b] Strains isolated from chicken intestine in Nakhon Ratchasima, Thailand: BC, *Salmonella* sp. enriched using RV broth and isolated on XLD agar; C, non-*E. coli* bacteria isolated on EMB agar; CM, *Salmonella* sp. isolated on mCCDA; CP, *Cl. perfringens* ; E: *E. coli*; L, non-*Listeria* bacteria isolated on PALCAM agar; RV, non-*Salmonella* bacteria enriched using RV broth and isolated on XLD agar ; S: *Salmonella* sp. enriched using TT broth and isolated on XLD agar; TT, non-*Salmonella* bacteria enriched using TT broth and isolated on XLD agar

[c] Strains isolated from food in Khon Kaen, Thailand: CP, *Cl. perfringens*; Sh, *Shigella* sp.

The biochemical characteristics of *E. coli* isolate E6 were the same as the reference strains. But for *E. coli* isolates E3 and E7, some difference characteristics including no gas production from utilization of rhamnose and negative xylose utilization were found, respectively. Almost all biochemical profiles of *Salmonella* sp. isolates were similar to the reference strains except that some isolates was able to

slowly hydrolyze gelatin (S2) and different properties for myo-inositol utilization. All biochemical characteristics of *Cl. perfringens* isolates were similar except for isolate CP3 which was unable to hydrolyze gelatin (Chapter 2). All target bacteria except for *Cl. perfringens* were grown on trypticase soy agar (TSA) (Appendix I, M1.6) at 37^{o}C for 24-48 h. For the cultivation of *Cl. perfringens*, the bacterium was cultured on tryptose sulphite cycloserine agar (TSC) (Biomark, Pune, India) containing egg yolk emulsion (Biomark) and incubated under anaerobic condition at 37^{o}C for 24 h.

3.2.2 Primer and probe design

For oligonucleotide array, the 16S rRNA genes specific for each bacterial species and genus were used as target regions. The full length nucleotide sequences of the 16S rDNA from 6 foodborne pathogens including 5 sequences from *E. coli*, 21 sequences from *Campylobacter* spp., 8 sequences from *Cl. perfringens*, 13 sequences from *L. monocytogenes*, 26 sequences from *Salmonella* spp., and 3 sequences from *Staph. aureus* were used. The sequences were downloaded from NCBI database. Nucleotide sequences were aligned using MegAlign DNAStar lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA) to obtain consensus sequences of each pathogen. Universal 16S rDNA primers were designed using Primerselect DNAStar lasergene 7 (DNASTAR Inc., Madison, Wisconsin, USA) based on the consensus sequences which were conserved for all target bacteria and contained variable regions in the PCR products. For *prf*A gene amplification, primers were designed based on the conserved regions of this gene which could be amplified from all *L. monocytogenes*. Sequences of the forward and reverse primers for 16S rRNA, *prf*A gene amplification are shown in Table 3.2. The sequences of variable region from the 16S rDNA and *prf*A were then used for probe design. Probes specific for each pathogen were

designed based on the variable regions of the 16S rDNA and conserved regions of *prf*A gene using the PICKY oligonucleotide design program (Chou et al., 2004).

Table 3.2 PCR primer sequences designed from this study for amplification of specific target genes

Target region	**Sequence (5' to 3')**	**Amplicon size (bp)**	**Reference**
Eubacteria 16S rRNA gene	**F**: AGACTCCTACGGGAGGC **R**: GGTAAGGTTCTTCGCGT	625-655	This work
L. monocytogenes *prf*A gene	**F**: CACAAGAATATTGTATTTTTCTATATGAT **R**: CAGTGTAATCTTGATGCCATCA	398	This work

3.2.3 Target gene amplification

Genomic DNA from 16-24 h grown pure cultures on TSA (Appendix I, M1.6) or TSC (Biomark) were extracted using the simple protocol of phenol-chloroform based method (Kumar et al., 2008). Briefly, bacterial cells were harvested from TSA or TSC agar and 250 µl of lysozyme solution (2.5 mg/ml in 10 mM Tris-Cl, pH 8) was added. The mixture was incubated at 37^{o}C for 2 h and the 500 µl of lysis buffer (50 mM Tris-Cl, pH 8, 100 mM EDTA, 1% SDS, and 1 mg/ml protenase K) was added and incubated at 50^{o}C for 30 min. Cell debris and protein were removed by adding 500 µl of phenol: chloroform: isoamyl alcohol solution (25: 24: 1) and

centrifuged at 14,000 rpm for 15 min. Genomic DNA in aqueous phase was precipitated using 125 mM NaCl and 1 volume of absolute isopropanol. The DNA pellets were washed in 70% ethanol and dry at room temperature. Finally, DNA pellets were then resuspended in 10 mM Tris-Cl containing 1 mM EDTA (TE), pH 8 and 10 µg/ml RNaseA. Genomic DNA obtained from this step was used as templates for 16S rRNA and specific genes amplification.

The genomic DNAs were used as template for amplification of the target genes. The PCR reactions were performed individually in a total volume of 25 µl containing 1× GoTaq Flexi buffer (Promega, Madison, USA), 1 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.4 µM of each forward and reverse primer (Table 3.2), 0.5 U GoTaq Flexi DNA polymerase (Promega), and 100 ng DNA templates. The PCR reactions were heated at 95^{o}C for 3 min and then, 35 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 60 s followed by a final step of 5 min incubation at 72°C. The PCR products were analyzed by electrophoresis on agarose gels (1%, w/v) and purified using QIAquick PCR Purification Kit (Qiagen, GmbH, Germany). The concentration was measured by Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA).

3.2.4 Oligonucleotide array preparation and detection

Nylon membranes (Roche, Mannheim, Germany) were used as the array matrix. Single strand (100 µM) probes were heated at 95^{o}C for 5 min and 2 µl were spotted on a specific position on the dry nylon membrane. The membranes spotted with probes were exposed to UV for 3 min to allow cross-linking of the probes onto the nylon membrane. Then the membrane were air-dried and stored in plastic bags at room temperature until use.

Purified PCR products of 100 ng were labeled with digoxigenin (DIG) molecule using DIG High Prime (Roche). The PCR products were denatured at 99^{o}C for 10 min and quickly chilled on ice. The 5× Mix DIG-High prime (Roche) containing random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme and buffer components were added into the denatured DNA and incubated at 37^{o}C for 1 h. The reactions were stopped by heating at 65^{o}C for 10 min and used for hybridization with specific probes on nylon membrane.

Membranes with spotted probes were pre-hybridized in a pre-warmed DIG Easy hybridization solution (Roche) at 35^{o}C with gentle shaking for 30 min. Prior to hybridization, the labeled PCR products were heated to 99^{o}C for 5 min, then immediately cooled on ice. These labeled PCR products were then added to newly pre-warmed hybridization solution (Roche). The membranes were then hybridized in the DIG Easy hybridization solution (Roche) containing labeled PCR products at 35^{o}C with gentle rotating for 4 h. After hybridization, the membranes were washed twice for 5 min each in 2× SSC (Roche), 0.1% SDS at 25^{o}C, twice for 10 min each in 0.5× SSC (Roche), 0.1% SDS at 45^{o}C and briefly washed in washing solution (Roche) at room temperature. Then the membranes were incubated for 30 min in blocking solution (Roche). Hybridization signals were detected by incubating the membrane in antibody solution (anti-digoxigenin-AP 1:5000 (150 mU/ml)) (Roche) for 30 min and washed twice with washing buffer (Roche) for 30 min each. After 5 min equilibration in detection buffer (Roche), the membranes were then incubated in freshly prepared color substrate solution using NBT/BCIP (Roche) in an appropriate container in the dark without shaking during color development. After 18 h of incubation, the reactions were stopped by washing the membrane for 5 min with

sterile double distilled water. The resulting images were documented by scanning.

3.3 Results

3.3.1 Primer and oligonucleotide probe design

The primer designed for the 16S rRNA genes indicated that the location of the forward and reverse primers related to the published *E. coli* 16S rDNA accession number EU337124 were 338 to 354 and 970 to 986, respectively. These sequences are conserved among all 5 pathogens (*E. coli*, *Cl. perfringens*, *L. monocytogenes*, *Staph. aureus*, *Salmonella* spp.) used in this study (Figure 3.3). Alignment results and positions of forward and reverse primers for amplification of *prf*A gene are shown in Figure 3.4. After amplification by these 16S rDNA universal primers, the PCR products containing variable regions of each target bacteria were obtained. However, sequence alignment of the 16S rDNA region indicated that differentiation of each target bacteria using variable region can be done only in the genus level.

The validation of 16S rDNA primer were done with several reference and isolated strains of bacteria. The products of 620-650 bp were obtained from PCR amplification using 16S rDNA primers from all target bacteria (Figure 3.5A). All 16S rDNA PCR products were purified (Figure 3.5B) and used as the template for DNA labeling process in the next step. In the case of *prf*A gene amplification, *prf*A gene was amplified from only *L. monocytogenes* DSM 12464 but not from *Listeria* sp. JCM 7679, *L. innocua* DSM 20649 or non-*Listeria* bacteria (Figure 3.6). All PCR products including 16S rRNA and *prf*A gene fragments were purified and labeled with DIG molecule and used for DNA hybridization.

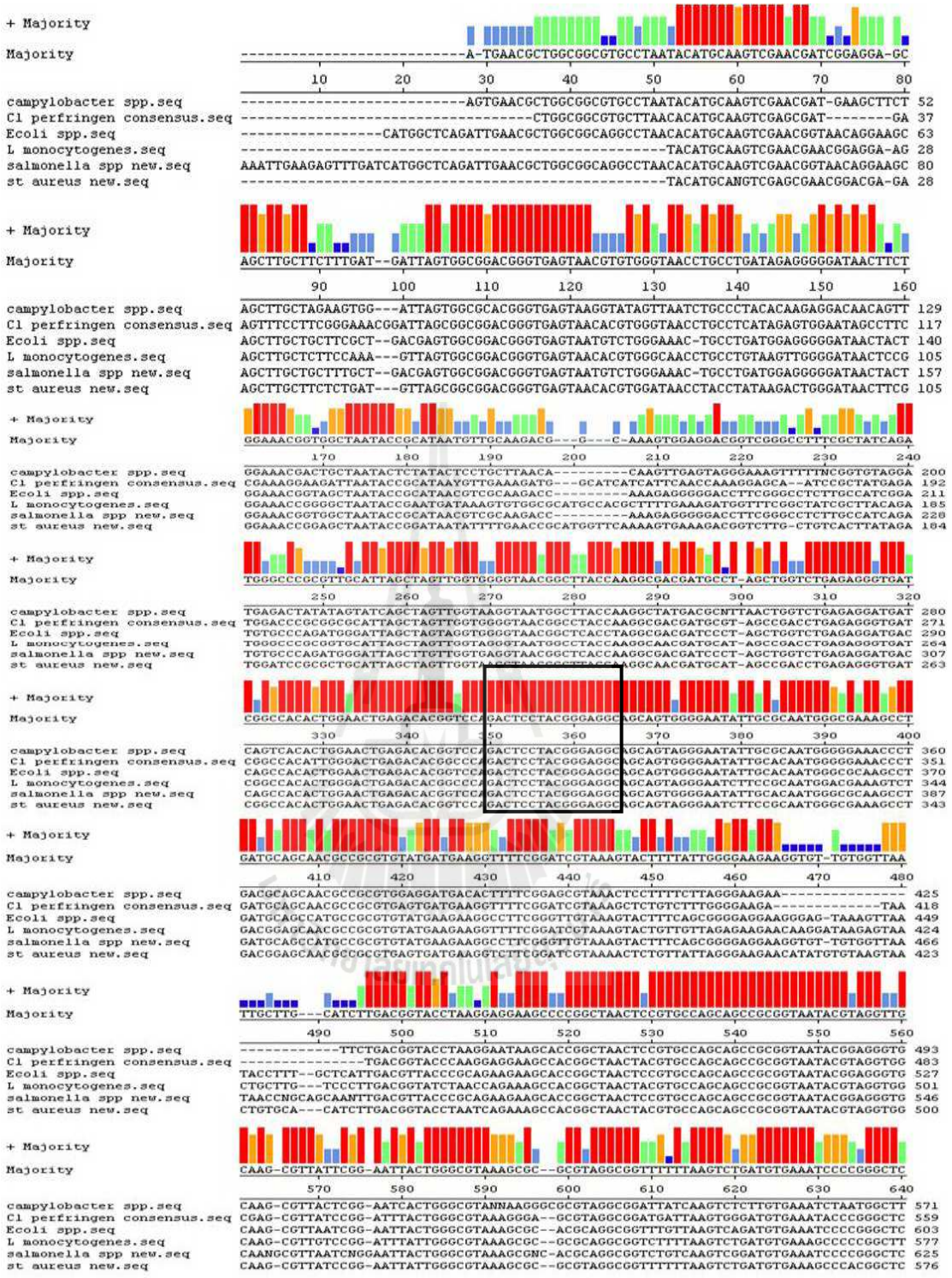

Figure 3.3 Alignment of 16S rRNA gene from 6 foodborne pathogens including *E. coli*, *Campylobacter* spp., *Cl. perfringens*, *L. monocytogenes*, *Salmonella* spp. and *Staph. aureus* using MegAlign DNAStar lasergene 7. The primers used for 16S rDNA amplification are boxed.

Figure 3.3 Alignment of 16S rRNA gene from 6 foodborne pathogens including *E. coli*, *Campylobacter* spp., *Cl. perfringens*, *L. monocytogenes*, *Salmonella* spp. and *Staph. aureus* using MegAlign DNAStar lasergene 7. The primers used for 16S rDNA amplification are boxed (Continued).

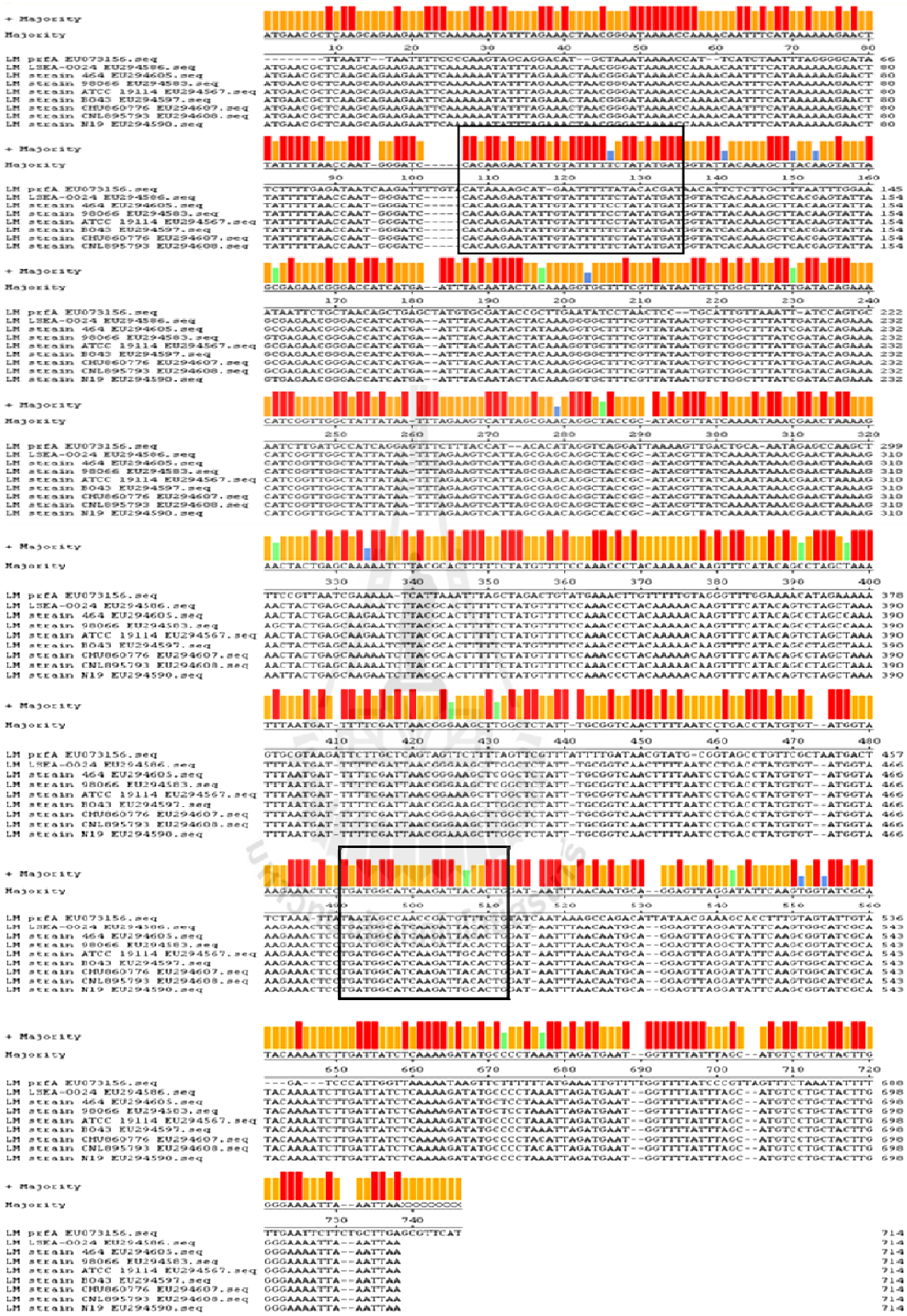

Figure 3.4 Alignment of *prf*A gene from *L. monocytogenes*. Positions of forward and reverse primers are boxed.

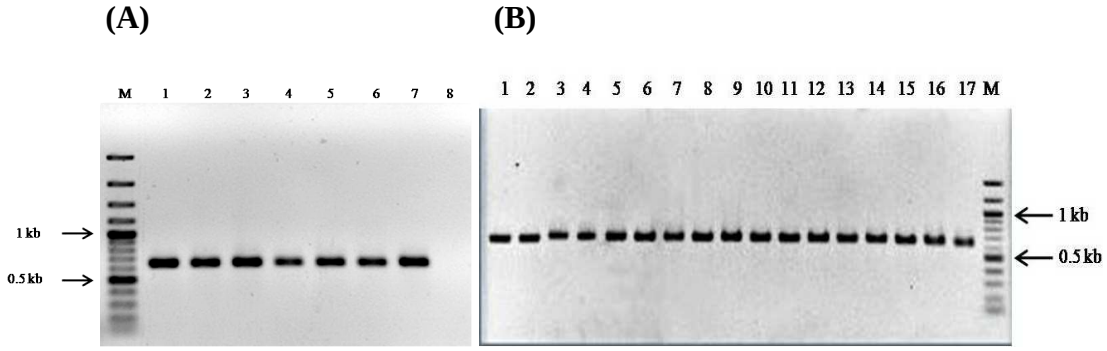

Figure 3.5 **(A)** Amplification of a part of the partial sequence of 16S rRNA gene using 20 ng bacterial genomic DNA as templates. Lanes: M, 100 bp DNA marker (Fermentas); 1, *E. coli* TISTR 887; 2, *S.* Typhimurium TISTR 292; 3, *S.* Enteritidis JCM 1652; 4, *Listeria* sp. JCM 7679; 5, *L. innocua* DSM 20649; 6, *Shigella* sp. isolated from food sample, Thailand; 7, *Staph. aureus* TISTR 517; 8, H_2O (negative control). **(B)** Purified 16S rDNA PCR products (100 ng) from different bacteria for post-PCR labeling process. Lanes: 1, *S.* Enteritidis JCM 1652; 2, *S.* Enteritidis TISTR 2394; 3, *S.* Typhimurium TISTR 292; 4-5, *Salmonella* sp. isolates S2 and S3, respectively; 6, *E. coli* TISTR 887; 7-9, *E. coli* isolates E3, E6 and E7, respectively; 10, non-*Salmonella* bacteria isolate RV3, 11, *Shigella* sp. isolate Sh1; 12, *Staph. aureus* TISTR 517; 13, *Listeria* sp. JCM 7679; 14, *L. monocytogenes* DSM 12464; 15, *L. innocua* DSM 20649; 16-17, *Cl. perfringens* isolates CP3 and CP5, respectively; M, 100 bp DNA marker (NEB).

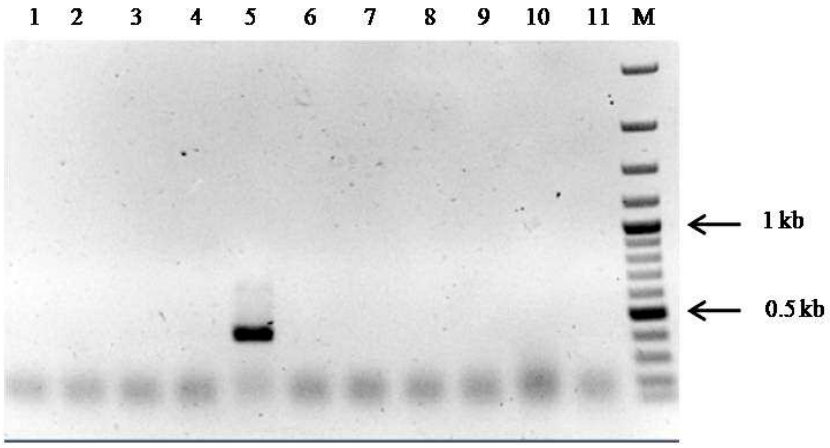

Figure 3.6 The amplification of *prf*A gene using 20 ng of genomic DNA of different bacteria as the templates. Lanes: 1, *Staph. aureus* TISTR 517; 2, *S.* Typhimurium TISTR 292; 3, *S.* Enteritidis TISTR 2394; 4, *S.* Enteritidis JCM 1652; 5, *L. monocytogenes* DSM 12464; 6, *Listeria* sp. JCM 7679; 7, *L. innocua* DSM 20649; 8, *Shigella* sp. isolate Sh1; 9-10, uncharacterized colony on TSA agar; 11, H_2O (negative control); M, DNA marker 100 bp (NEB).

The sequences of amplified products were then used for probe design using PICKY oligonucleotide design program (Chou et al., 2004). Probes obtained from this step are shown in Table 3.3. All probes were tested for their specificity using DNA extracted from pure culture of reference and isolated strains of the target bacteria. Some of the probes designed using PICKY program in this experiment were found to be similar to the probes designed by Chiang and colleagues (2006) and Mao and colleagues (2008) and one of the *L. monocytogenes* 16S rDNA probe (LM2) was exactly the same as one of the probe from Mao and colleagues (2008). To confirm that the probes obtained from this investigation can be used for pathogen detection,

the probes (Table 3.3) were tested for hybridization with the target bacteria of interests.

Table 3.3 Sequences of the 16S rDNA and *prf*A probes spotted on the oligonucleotide array

Species	**Target region (gene)**	**Probe sequences (5' to 3')**	**References**
Cl. perfringens	16S rRNA	CP 1: AAGCTCTGTCTTTGGGGAAGATAATGACGG CP 2: ACGATGAATACTAGGTGTGG CP 3: TCCAAACTGGTTATCTAGAGTGCA CP 4: GGCGGATGATTAAGTGGGATGT CP 5: AGATTAGGAAGAACACCAGT	This work This work This work Mao et al.(2008), This work This work
E. coli	16S rRNA	EC 1: AGGAAGGGAGTAAAGTTAATACCTTTGCT EC 2: CTGCATCTGATACTGGCAAG	Chiang et al. (2006), Mao et al. (2008), This work This work
Salmonella spp.	16S rRNA	SM 1: AGGAAGGTGTTGTGGTTAATAAC SM 2: TCTGTCAAGTCGGATGTGAA	This work Chiang et al. (2006), This work
Staph. aureus	16S rRNA	SA 1: AGAACATATGTGTAAGTAACTGTGC SA 2: CGCAGAGATATGGAGGAACA	Mao et al.(2008), This work This work
L. monocytogenes	16S rRNA	LM 1: GCTTGTCCCTTGACGGTATCTAACC LM 2: GTTTTCGGATCGTAAAGTACTGTTGTTAGAGA	This work Mao et al.(2008)
Campylobacter spp.	16S rRNA	CM 1: AGGCAGATGGAATTGGTGGTGTAGG CM 2: AGCGTAAACTCCTTTTCTTAGGGA	This work This work

Table 3.3 (Continued)

Species	**Target region (gene)**	**Probe sequences (5' to 3')**	**References**
L. monocytogenes	*prf*A	prfA 2: ACAAAGGTGCTTTCGTTATAATGTCTGGCT prfA 3: AGCTTACAAGTATTAGCGAGAACGGGACCA	This work This work

3.3.2 Oligonucleotide array for multiple foodborne pathogen detection

3.3.2.1 Oligonucleotide array optimization

Only the 16S rDNA probes of *E. coli*, *Salmonella* spp., and *Staph. aureus* were tested to obtain the optimum concentration for DNA hybridization system. The probes were spotted on nylon membrane at specific position as shown in Figure 3.7A at the amount of 100 and 200 pmol. The hybridizations were carried out with 100 ng DIG labeled target PCR products at 35^{o}C for 4 h. The hybridization results indicated that all hybridization signals were not different between 100 (Figure 3.7B (I)) or 200 pmol probes (Figure 3.7B (II)) except for the probe of *S.* Typhimurium. Hybridization signal of *S.* Typhimurium could be observed when the amount of probe was 200 pmol but not 100 pmol. All hybridization between probes and specific target in Figure 3.7B showed that no cross-reactivity was observed between the probes and the non-specific targets. To test for the probe specificity with other organisms, 200 pmol probes were used.

(A)

SM 1	SM 2	SA 1	SA 2
P		16S	
EC 1	EC 2		

(B)

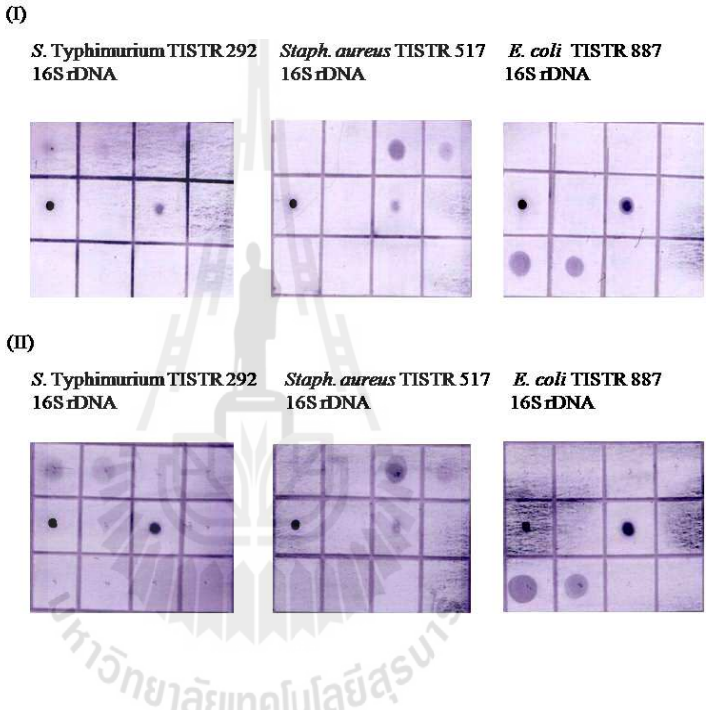

Figure 3.7 Optimization of oligonucleotide array for foodborne pathogen detection. **(A)** Position of specific probes on nylon membrane. Positive controls are 0.1 ng of DIG-label control DNA (pBR328 DNA, linearized with *Bam* HI) (P) and 100 pmol 16S rDNA forward primer (16S). The abbreviated letters in grids are probe names as shown in Table 3.3. **(B)** Hybridization patterns of specific probes and labeled target PCR products. Probes were spotted on nylon membrane at 100 pmol (I) and 200 pmol (II). The target DNA were 16S rRNA gene from each bacterium as labeled on the top of each blot.

3.3.2.2 Probe validation and specificity test

Specific detections of several bacteria were performed by hybridization of labeled 100 ng of 16S rDNA and *prf*A PCR product with 200 pmol probes. Each labeling target PCR product was hybridized individually with the nylon membrane containing specific probes of 16S rRNA gene at specific positions as shown in Figure 3.8A. The hybridization patterns between specific probes of the 16S rRNA genes with their specific targets are shown in Figure 3.8B. The amplification was done with total of 31 bacterial strains (Table 3.1) with single primer pair and the accuracy of each was evaluated as summarized in Table 3.4. The specific hybridization patterns between the specific probes with their specific targets were found in *Cl. perfringens* (Figure 3.8B (21)-(24)), *Listeria* spp. (Figure 3.8B (25)-(27)), and *Staph. aureus* (Figure 3.8B (31)). In the case of *L. monocytogenes* specific detection, strong hybridization signals were found with probes LM1 and LM2 (Figure 3.8B (25)-(27)). These results demonstrated that 16S rRNA genes of *Listeria* genus were highly conserved among *Listeria* species. The probe LM1 was highly conserved among *Listeria* species with high accuracy (Table 3.4, 100% accuracy) for specific detection of *Listeria* genus. To detect *L. monocytogenes* in the species level, *prf*A gene was used as target gene for specific detection. In this research, *prf*A gene was able to be amplified from *L. monocytogenes* DSM 12464 but not from *L. innocua* DSM 20649. After DNA labeling process using amplified product of *prf*A gene as template, the labeled products were able to specifically hybridize to the prfA probes (Table 3.4, 100% accuracy) with no-cross reactivity with the other 16S rDNA probes on the array (Figure 3.8B (28)). Thus specific detection of *L. monocytogenes* can be performed using hybridization of the 16S rRNA gene and *prf*A gene for detection in

the genus and species level, respectively. However, the cross-reactions of the 16S rDNA PCR products from *Cl. perfringens* with the LM2 probe (85% accuracy) (Table 3.4) were found in all *Cl. perfringens* isolates (4 isolates) (Figure 3.8B). For *Cl. perfringens*, strong hybridization signals were observed from probes CP1, CP3, CP4 and *Listeria* probe (LM2) (Figure 3.8B (21)-(24)). However, the hybridization patterns of *Cl. perfringens* and *Listeria* spp. were different and could be differentiated.

For *Salmonella* spp. and *E. coli*, hybridization patterns of these bacteria were similar from both reference and isolated strains. Accuracy of the *Salmonella* and *E. coli* probes were ranging from 77-95% (Table 3.4). Similar hybridization patterns of *E. coli* and *Shigella* sp. were found (Figure 3.8B (1)-(4), (32)). Moreover, cross-reactivities of *E. coli* probes EC1, EC2 with isolate RV3 (Figure 3.8B, (19)) were also observed. Biochemical profiles of the isolate RV3 was similar to *E. coli* excepted that this isolate was unable to utilize lactose. In the case of *Salmonella* detection, cross-reactivities of the *Salmonella* probes, SM2 with the non-target bacteria isolates C2, C4, RV2, L2 (Figure 3.8B (5), (7), (18), (30)) and probes SM1, SM2 with the non-target bacteria isolate C3 (Figure 3.8B, (6)), were observed. The identification of C2, C3, C4, RV2, and L2 using several biochemical reactions indicated that these bacteria were Gram–negative, and identified as belonging to either non-*Salmonella* or non-*Listeria* or non-*E. coli* bacteria (Chapter 2). These results showed that *Salmonella* and *E. coli* probes cross-react with the non-*Salmonella* and non-*E. coli* bacteria from the enrichment culture of *E. coli*, *Listeria* spp. and *Salmonella* spp. Therefore, the *E. coli* and *Salmonella* spp. probes targeted to the 16S

rRNA gene only were not specific enough to detect these bacteria directly from the enrichment culture.

Table 3.4 Probe specificity test using isolated and reference strains of target and non-target bacteria

Target bacteria	**Probe name**	**Number of target bacterial strains (T)**		**Number of non-target bacterial strains (NT)**		**% of accuracy** [a]
		Number of tested strains	**Positive signal detection (T^+)**	**Number of tested stains**	**Positive signal detection (NT^+)**	
Clostridium perfringens	CP 1	4	4	27	0	100
	CP 2	4	0	27	0	0
	CP 3	4	4	27	0	100
	CP 4	4	4	27	0	100
	CP 5	4	0	27	0	0
Escherichia coli	EC 1	4	4	27	2	92.6
	EC 2	4	4	27	2	92.6
Listeria spp.	LM 1	3	3	28	0	100
	LM 2	3	3	28	4	85.7
Salmonella spp.	SM 1	9	9	22	1	95.5
	SM 2	9	9	22	5	77.3
Staphylococcus spp.	SA 1	1	1	30	0	100
	SA 2	1	1	30	0	100
L. monocytogenes	prfA 2	1	1	30	0	100
	prfA 3	1	1	30	0	100

[a] % Accuracy = $[(T^+ \times 100)/ \text{No. T}] - [(NT^+ \times 100)/ \text{No. NT}]$

(A)

SM 1	SM 2			SA 1	SA 2
		CP 1	CP 2	CP 3	CP 4
	P		16S		CP 5
		EC 1	EC 2		
LM 1	LM 2			CM 1	CM 2
		prfA 2	prfA 3		

(B)

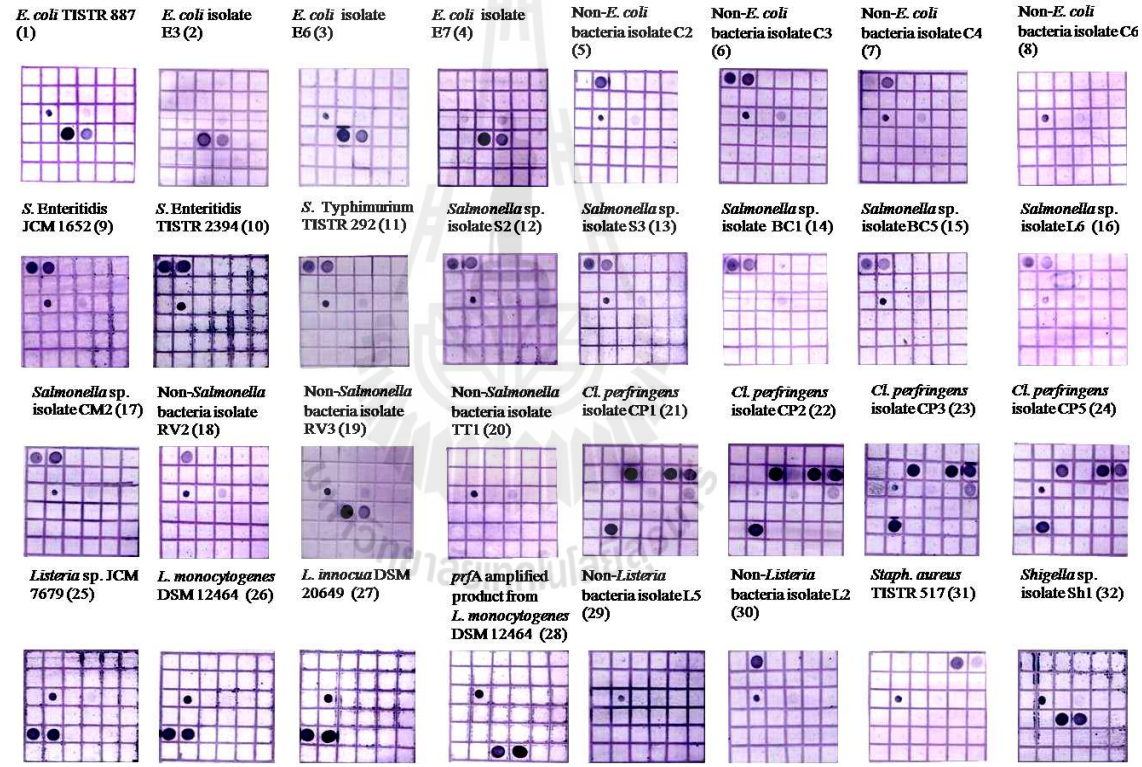

Figure 3.8 Probe validation and specificity test for foodborne pathogens detection. **(A)** Position of specific probes on nylon membrane. Positive controls are 0.1 ng of DIG-labeled control DNA (pBR328 DNA, linearized with *Bam* HI) (P) and 100 pmol 16S rDNA forward primer (16S). The abbreviated letters in grids are probes names as shown in Table 3.3. **(B)** Hybridization patterns of target and non-target bacteria detected by naked eyes on oligonucleotide array. The target DNA was 16S rDNA or *prf*A gene fragment from each bacterium as labeled on the top of each blot.

In this investigation, no cross-reactivity of the labeled 16S rDNA PCR products with probes on the array was found from the three non-target bacteria isolates C6, TT1 and L5 (Figure 3.8B ((8), (20) and (29), respectively). The results also showed that the hybridization of *Cl. perfringens* and *Staph. aureus* could be differentiated from *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp. and other non-target bacteria in enrichment culture using only the 16S rRNA gene as target.

3.3.2.3 Multiple target bacteria detection using oligonucleotide array based method

For multiple target bacteria detection, only 16S rRNA gene was used as the target. Genomic DNA from 3, 4 and 5 target bacterial species were mixed at the concentration of 1 ng each and used as templates for the 16S rDNA amplification. Two hundred ng of the PCR products were labeled and hybridized with specific probes on the spotted nylon membrane. Results of several target bacteria detected by oligonucleotide array are shown in Figure 3.9. These results indicated that our detection system can be used to detect several target bacteria of at least 1 ng in mixed genomic DNA from the 5 bacterial species using oligonucleotide array. Based on the results of this research, the oligonucleotide array hybridization targeted to the 16S rRNA gene using a single primer pair was suitable for the first step of multiple target bacterial screening in short time. The positive results from oligonucleotide array should be further confirmed by specific gene hybridization. Alternatively, this method could be used to identify presumptive pure colony after the isolation step which would save considerable amount of time compare to the normal conventional biochemical tests.

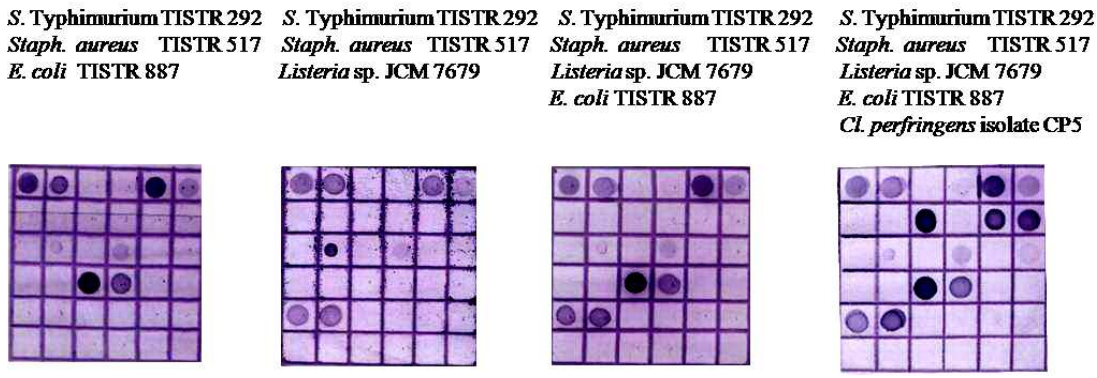

Figure 3.9 Multiple target bacteria detection by oligonucleotide array hybridization based method. Each specific probe (200 pmol) was spotted on nylon membrane at the same position as shown in Figure 3.8A. The target DNA was 16S rRNA gene from each bacteria.

3.4 Discussion

Conventional methods for detecting foodborne pathogens involved separate culture steps followed by biochemical identification and serotyping. These methods are cumbersome and time consuming. Therefore, rapid, specific, and sensitive methods for detecting and identifying pathogens have been developed. Rapid detection and identification of several foodborne pathogens are the key issues for diagnosis, treatment and timely control of foodborne infections (Hong et al., 2004; You et al., 2008).

Advantages of oligonucleotide array for multiple pathogens detection are that the multiple target bacteria can be detected in a single reaction. By these methods labor, cost, and identification time can be reduced. In this part, oligonucleotide array

targeted to 16S rRNA and *prf*A genes were developed and evaluated for specific detection of dominant foodborne pathogens and food safety indicator in chicken meat including *Cl. perfringens*, *L. monocytogenes*, *Salmonella* spp., *Staph. aureus*, and *E. coli*. For specific detection of *Salmonella* spp. and *E. coli* using 16S rRNA gene as target, hybridization patterns were similar from both reference and isolated strains. Moreover, the hybridization patterns of all isolates of *Salmonella* sp., *E. coli* and *Cl. perfringens* were similar among each pathogen. Although the variation properties from each strain were found but probes design in this research can be used for specific detection of these foodborne pathogens. These results indicated that the 16S rDNA target regions were conserved among each bacterial pathogen.

Detection of multiple target bacteria by 16S rRNA gene oligonucleotide array hybridization showed that the identification can be done at the genus level. Some cross-reactivities were found in the non-target bacteria isolated from the enrichment culture (Figure 3.8B). Cross-reactions were also found in *Salmonella* spp. and *E. coli* when 23S rRNA gene were used as target (Hong et al., 2004), *E. coli* and *Shigella* spp. (Chiang et al., 2006; Hu et al., 2011) when 16S rRNA and *gro*EL genes were used. In the case of *E. coli* and *Salmonella* spp., although the identity of this 16S rDNA region was 95% but this research was able to differentiate them.

In this investigation, the same results of cross-reaction were found in *E. coli* and *Shigella* spp. but *E. coli* and *Salmonella* spp. can be differentiated using 16S rRNA gene as target. In 2006, Chiang and colleagues reported that genus *Shigella*, which is closely related to *E. coli*, generated the same hybridization patterns using the 16S rDNA oligonucleotide array. The classification of these closely related species of *Shigella* spp. and *E. coli* is difficult to achieve through 16S rRNA gene analysis

because *Shigella* and *E. coli* have been considered to be a single species, based on DNA homology (Fukushima et al., 2002). Probe sequence with homology higher than 85% to other bacteria may cross-react with strains of these closely related species (Chiang et al., 2006). Therefore, species-specific gene is needed for differentiation between *Shigella* and *E. coli*.

In the case of *prf*A gene amplification, *prf*A gene was specific for only *L. monocytogenes* DSM 12464 (Figure 3.6). The *prf*A gene product regulates the expression of listeriolysin which is a major virulence factor expressed by pathogenic *Listeria* spp. (Wernars et al., 1992). The amplification of *prf*A gene with primers designed in this work was specific for *L. monocytogenes* and no-cross reactivity with other bacteria was observed. This result indicated that *prf*A gene was suitable for specific detection of *L. monocytogenes* and also used as target gene in next part.

In this investigation, different hybridization signal from different probes were observed. For hybridization of *Cl. perfringens*, the hybridization patterns observed from all *Cl. perfringens* isolated strains were similar (Figure 3.8B (21-24)). These results indicated that the DNA sequence of the target region derived from *Cl. perfringens* was conserved thus similar hybridization patterns were found from all isolates. Hybridization of labeled 16S rDNA of *Cl. perfringens* and the LM2 specific probes for *L. monocytogenes* (which is the same as one of the probe from Mao et al. (2008)) showed strong signals. These results indicated that LM2 probe is not suitable for specific detection of *L. monocytogenes* since it cross hybridized with the 16S rDNA from *Cl. perfringens*. Although strong signals were observed from CP1, CP3, and CP4 when 16S rRNA gene of *Cl. perfringens* was used as target but very weak signal were detected from probes CP2 and CP5 (Figure 3.8B (21)-(24)). In this study,

the 16S rDNA probes were designed to hybridize with complementary strands of their specific target pathogen at difference positions. Thus, it might be possible that the differences in hybridization signal seen from each probe positions of *Cl. perfringens* could be due to the differences of the probe locations on the specific DNA target. The secondary structure of DNA template and oligonucleotide probe position on the target DNA template can influence the hybridization signals (Matveeva et al., 2003; Peytavi et al., 2005; Franke-Whittle et al., 2006).

The evaluation of some published probes for specific detection of *E. coli*, *L. monocytogenes*, and *Salmonella* spp. were also investigated. The amplification regions targeted to 16S rRNA gene in this investigation was similar to Chiang and colleagues (2006) and Mao and colleagues (2008). However, some probes designed were different from previous research since different target bacterial groups were used for probes design. The probes LM2 (Mao et al., 2008), EC1 and SM2 (Chiang et al., 2006) cannot be used to differentiate *E. coli*, *L. monocytogenes*, *Salmonella* spp. because cross-reactivity with closely relate bacteria and non-target bacteria from the enrichment culture were found. These results indicated that the probe validation and specificity test is very important for bacterial identification by oligonucleotide array based methods.

The strong and weak points of oligonucleotide array for multiple pathogen detection were evaluated in this study. The advantage of developed oligonucleotide array in this investigation showed that the same size PCR product can be differentiated by specific probes hybridization. Compared to PCR based methods, detectability of the PCR based methods was lower than that of oligonucleotide array hybridization (Hong et al., 2004; Chiang et al., 2006). Multiple pathogen detection at

least 5 various bacterial target species can be done with easy result interpretation and easy detection systems by naked eyes. No complicate system or expensive equipment needed for signal detection because strong hybridization signal can be observed using post-PCR labeling of the target regions. This method could be used to detect the pure presumptive colonies of interest after the isolation step. However, weak point of this method was that the use of the 16S rRNA gene as target was not enough to directly detect foodborne pathogens especially *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *Shigella* spp. from enrichment culture. The isolation of bacteria from chicken intestine samples (Chapter 2) showed that *Salmonella* sp. and other non-target bacteria were able to grow and frequently found in enrichment culture broths supplemented with antibiotic for cultivation of *Listeria* spp. and *Campylobacter* spp. Thus genes specific to each pathogen are required for oligonucleotide array result confirmation. To solve the problem of cross-reaction of oligonucleotide array target to 16S rRNA gene, specific gene amplification by m-PCR were performed in next part.

3.5 Conclusions

In conclusions, the labeled target regions of the 16S rDNA and *prf*A amplificons generated by post-PCR labeling methods could be successfully used for the differentiation of target bacteria in the genus and species levels, respectively. Steps of probe selection were done by post-PCR labeling methods. The detection of foodborne pathogens including *E. coli*, *Cl. perfringens*, *L. monocytogenes*, *Staph. aureus*, and *Salmonella* spp. by oligonucleotide array were possible and this method is suitable for probe selection. This oligonucleotide array based method could improve the accuracy of the bacterial detection by PCR technique. In the PCR reactions,

similar size of PCR products containing different population could not be differentiated. Thus downstream technique such as DNA hybridization based method shown in this research could be applied to overcome this limitation. By this detection system, several target organisms can be easily detected and correctly interpreted. In addition, this method could be used to identify the presumptive colonies of interest after the isolation step with considerable time saving in comparison to the biochemical method. Thus identification system in this research could be used as a rapid alternative method to the biochemical confirmation. In next part, to avoid the false positive results of multiple pathogen detection in high bacteria background samples, oligonucleotide array targeted to 16S rRNA gene were combined with amplification of other specific genes by m-PCR or conventional PCR. These combined methods should be a suitable system for specific detection of multiple target bacteria directly in enrichment culture.

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CHAPTER IV

DEVELOPMENT OF OLIGONUCLEOTIDE ARRAY TO SPECIFICALLY DETECT DOMINANT BACTERIAL FOODBORNE PATHOGENS IN CHICKEN MEAT

Abstract

In this study, oligonucleotide arrays were developed to simultaneously determine the presence of 3 dominant foodborne pathogens and 1 microbial food safety indicator in fresh chicken meat. Multiplex PCR (m-PCR) or conventional PCR were combined with oligonucleotide array assays for specific detection of *Escherichia coli*, *Listeria monocytogenes, Salmonella* spp., and *Shigella* spp. Moreover, *Clostridium perfringens* and *Staphylococcus aureus*, the regulated foodborne pathogens in poultry meat, were also tested to evaluate the specificity of these assays. Probes targeted to 16S rRNA and species or genus specific genes including *usp*A, *prf*A, *fim*Y, and *ipa*H genes were selected for specific detection of *E. coli*, *L. monocytogenes, Salmonella* spp., and *Shigella* spp., respectively. The combination of m-PCR or conventional PCR and oligonucleotide array revealed discriminatory power among genera and species of all 4 target bacteria with low or no incidence of false negative results. Target genes amplification by m-PCR or conventional PCR follow by oligonucleotide array was able to distinguish all 4 target bacteria with a detection

sensitivity of 1 ng or 0.1 ng of each genomic DNA, respectively. The oligonucleotide array was also applied to 4 fresh chicken meat samples with the target bacterial spiked and non-spiked. Target bacterial enrichment and DNA amplification by conventional PCR or m-PCR were performed prior to oligonucleotide array hybridization. The validation of conventional PCR-oligonucleotide assay demonstrated that all 4 target bacteria could be detected simultaneously in fresh chicken meat samples with a detection sensitivity threshold of at least 3 and 10 cells for detection of *Sh. boydii* and *L. monocytogenes* in 25 g sample, respectively. The application of m-PCR couple with oligonucleotide array could simultaneously detect 3 target bacteria including *E. coli*, *L. monocytogenes*, and *Salmonella* sp. from fresh chicken meat with a detection sensitivity of at least 10 cells of *L. monocytogenes* in 25 g sample. However, combining the PCR and oligonucleotide array methods could enhance accuracy, sensitivity, and lower the times required for foodborne pathogen detection and identification compared to conventional culture. These combinations could also solve the minor problem of cross-reactivity from the non-target bacteria isolated from the enrichment culture in the oligonucleotide array hybridization and the difficulty in results interpretation of the m-PCR or conventional PCR detection.

4.1 Introductions and review literature

Foodborne diseases are some of the most widespread health problems in the world. Regulations for foodborne pathogens include *E. coli*, *Salmonella* spp., *Staph. aureus*, *L. monocytogenes*, *Cl. perfringens*, *Camp. jejuni*, and *Camp. coli* in poultry meat are required (Mulder and Hupkes, 2007). As review in Chapter 2, the prevalence

of the foodborne pathogens and microbial food safety indicators in poultry especially *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* have been reported in Thailand and many countries (Sackey et al., 2001; Bangtrakulnonth et al., 2004; Angkititrakul et al., 2005; Nierop et al., 2005; Padungtod and and Kaneene, 2005; Cortez et al., 2006; Lekroengsin et al., 2007; Vindigni et al., 2007; Minamia et al., 2010; Stonsaovapak et al., 2010). Therefore, detection of these organisms with rapid, sensitive, and easy methods is required. The molecular based methods such as oligonucleotide array and multiplex PCR (m-PCR) have been applied to detect multiple pathogens for time and labor saving (Yoo et al., 2004; Nugen and Baeumner, 2008).

4.1.1 Oligonucleotide array for multiple target bacteria detection

As review in Chapter 3, an essential feature of the DNA array technique is the hybridization of labeled DNA fragments with arrays of immobilized probes (Gauthier and Blais, 2003). This method can be applied for multiple pathogens and microbial community detection in food samples. Consensus gene among many pathogenic and target bacteria which can be amplified by a single pair of universal primer has been used (Hong et al., 2004; Chaing et al.,2006; Wang et al., 2007; Mao et al., 2008; Giannino et al. 2009; Hu et al., 2011). Because a lot of information of the 16S rDNA nucleotide sequences from a number of bacteria is available which is good for probes design, therefore the 16S rDNA has been widely used as the target gene for bacteria detection (Lin and Tsen, 1999; Wang et al., 2002; Chiang et al., 2006; Franke-Whittle et al., 2006; Eom et al., 2007; Wang et al., 2007). Several publications have also reported about the limitation of the 16S rRNA gene regarding to its diversity (Chiang et al., 2006; Wang et al., 2007). In this research as reported in

Chapter 3, cross-reactions of some probes with non-target bacteria were also found. The cross-reactions of probes targeted to 16S rDNA were found in *E. coli*, *Shigella*, *Salmonella*, and *L. monocytogenes*. The results in Chapter 3 showed that the hybridization pattern of *Cl. perfringens* and *Staph. aureus* could be differentiated from *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp. and other non-target bacteria in enrichment culture using only the 16S rRNA gene as target. Therefore, the dominant foodborne pathogens in poultry including *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp. were used as the target bacterial model for the method development in this part. Results in Chapter 2 and 3 indicated that these bacteria showed cross-reactivity with probe design targeted to 16S rRNA of each other and they could be found as co-occurrence in the same enrichment culture medium. Therefore, species or genus specific genes are needed for simultaneous detection of these bacteria. The conventional PCR and m-PCR based method have been widely used and adapted for the rapid detection of single species or multiple bacterial species for these bacteria. Thus an alternative method of m-PCR targeted to specific genes for multiple pathogen detection was investigated in this part.

4.1.2 Specific gene for target bacteria detection

For single species detection, different target genes were used for detection of *E. coli*, *Salmonella* spp., and *Shigella* spp. by PCR and m-PCR. Specific detection of *E. coli* has been reported by Chen and Griffiths (1998) and Osek (2001). In 1998, Chen and Griffiths developed a PCR-based assay to differentiate generic *E. coli* from other Gram-negative bacteria using the primers derived from the DNA sequences flanking the gene encoding the universal stress protein (*usp*A). They found that the 884 bp region of *E. coli* chromosome can be amplified from all 45 *E. coli* isolates

tested include the non-pathogenic K-12 strains and the pathogenic VTEC reference cultures. The specificity of this target gene was investigated from 11 non-*E. coli* Gram-negative bacteria isolates tested. No amplified product was observed from the PCR amplification from any of the non-*E. coli* Gram-negative bacteria include *Sh. dysenteriae*. In 2001, Osek developed a m-PCR protocol using primer sets that directly detect genes that are characteristic of *E. coli* and all 3 important enterotoxin marker genes. They used *usp*A gene as an internal control in the m-PCR amplification with another primer specific gene for differentiation of each *E. coli* strains. They found that, the universal stress protein encoded gene is a highly conserved and presented in all *E. coli* bacteria. This information indicated that *usp*A is conserve among *E. coli*. Therefore, *usp*A gene was used as target gene for differentiation between *E. coli* and non-*E. coli* bacteria in my research.

For *Salmonella* spp. detections, the most commonly target genes used for specific detection of this organism are *fim*Y and *inv*A genes (Yeh et al., 2002; Cortez et al., 2006; Nierop et al., 2005; Salehi et al., 2005; Germini et al., 2009; Mao et al., 2008). Fimbriae are proteinaceous appendages on the outer membrane of most Enterobacteriaceae, including the *Salmonella* species. Type 1 fimbriae are the most common fimbriae in the *Salmonella* species. Three genes, *fim*Z, *fim*Y, and *fim*W, have been implied as regulatory genes of the major fimbrial subunit protein gene, *fim*A. The amino acid sequences of FimZ and FimW share a relatively high homology with those of the prokaryotic regulators in the Gen-Bank database, whereas FimY shares only a few homologies with other prokaryotic proteins in the database. FimY is more distinct from other regulatory proteins of the family Enterobacteriaceae. The unique characteristics of the *fim*Y gene make it a useful target for detecting *Salmonella*

species (Yeh et al., 2002). Therefore, *fim*Y is a promising candidate gene to detect *Salmonella* using a DNA-based diagnostic test (Yeh et al., 2002). In 2002, Yeh and colleagues evaluated the suitability of *fim*Y gene amplification by PCR for specific detecting of *Salmonella* species. In their research finding, *fim*Y is quite common among serovars of *Salmonella*. Two serovars of *Salmonella* serovars form type 1 fimbriae and type 2 fimbriae were tested for the present of this gene. Type 2 fimbriae exhibit different adhesive properties from type 1 fimbriae. Both *Salmonella* serovar showed amplicons of this gene after PCR amplification. These results indicating that *fim*Y is present among these *Salmonella* serovars.

Another gene which is widely used for specific detection of *Salmonella* spp. is *inv*A gene. The *inv*A-C locus is a part of a 40 kb region of the chromosome mapping at centrisome 63, which is absent in phylogenetic related bacteria such as *E. coli*. An estimate of 20 genes are in this region know as *Salmonella* pathogenic island I (SPI-1). The function of the invasion gene products seem to be related to flagella protein. SPI-1 encodes the specialized type III protein secretion apparatus. Type III secretion systems have unique features that differentiate them from other protein secretion systems. Of this system, more than 15 proteins are inserted in the inner and outer membrane (Portillo, 2000). The *inv*A gene, codes for protein in the inner membrane of bacteria, is required for the ability of this organism to invade cultured epithelial cells (Salehi et al., 2005). This gene is the first gene of an operon containing three or possibly more genes arranged in the same transcriptional unit. These genes have been shown to be present and function in most (if not all) *Salmonella* serotypes (Galán, et al., 1992).

In 2003, Malkawi and Gharaibeh evaluated a m-PCR for rapid and reliable detection of naturally *Salmonella enterica*-contaminated meat and poultry products. A random fragment of 429 bp specific to *Salmonella enterica* only, *omp*C, and *inv*A gene were used as targets in this m-PCR reaction. They showed that m-PCR was successful, specific and faster in detecting *Salmonella enterica* in food samples. However, they could not detect the amplicons of *omp*C in some food samples and in some serovar of *Salmonella* reference strains.

In 2005, Salehi and colleagues reported a PCR based method for identification of *Salmonella* isolated strains using *inv*A gene as target. Thirty *Salmonella* strains were isolated from broiler specimens and subjected to *Salmonella* specific-PCR using primers belong to *inv*A. All isolates including positive control and *S.* Arizona generated a single 284 bp amplified DNA fragment. Other PCR amplification from bacteria includes *Citrobacter freundii, Sh. boydii, Sh. sonnei, E. coli,* and *Proteus mirabilis* did not showed any amplified DNA fragment. They concluded that the PCR based method targeted to *inv*A gene was successfully used to confirm the *Salmonella* isolates with no non-specific amplification observed from non-*Salmonella* species. For another specific gene for *Salmonella* detection, De Freitas and colleagues (2010) developed a multiplex PCR to detect genus *Salmonella* and serotypes Enteritidis, Typhi and Typhimurium in refrigerated carcasses and chicken viscera. Specific genes including *omp*C, *sdf*I, *via*B, *spy* gene were used as targets for bacterial differentiation. They showed that the primers were specific to the target regions of their respective serotypes. No nonspecific reactions occurred with other serotypes. The protocols and primers used were effective for the amplification of a fragment of 204 bp of *omp*C gene for the genus *Salmonella*, a fragment of 304 bp

of the *sdf*I gene for serotype Enteritidis, a fragment of 738 bp of the *via*B gene for serotype Typhi, and 401 bp of the *spy* gene for serotype Typhimurium. *Salmonella* sp. detection both by m-PCR and conventional microbiological methods yielded similar results in poultry carcasses and viscera. In 2011, Silva and colleagues developed primers complementary to the *inv*A gene and sets of gene specific primers for identifying *Salmonella* serovar Enteritidis. The m-PCR assay developed in their study showed high sensitivity and specificity in the detection of *Salmonella* spp. and *S.* Enteritidis in chicken carcasses, minas cheese and fresh pork sausage, after 24 h enrichment in a non selective enrichment medium.

For *Shigella* detection, the *ipa*H and *vir*A genes were used as specific target genes in many research. Shigellae usually harbor various plasmids, such as those required for bacterial invasion into the host intestinal epithelial cells and antibiotic resistance (Na-Ubol et al., 2006). Genes required for entry of bacteria into epithelial cells and the induction of apoptosis in infected macrophages are clustered on a 30 kb region of the virulence plasmid (VP). This region encodes components of a type III secretion (TTS) apparatus, substrates of this secretion apparatus (the translocators and the effectors), their dedicated chaperones (IpgA, IpgC, IpgE and Spa15), and two transcriptional activators (VirB and MxiE) (Gall et al., 2005). During the process for colonization, *Shigella* deliver many different virulence determinants (more than 25) into host cells and the surrounding space through the type III secretion system (TTSS) **(**Ashida et al., 2007). The current model of the TTS pathway proposes that, upon contact of bacteria with host cells, translocators insert into the membrane of the host cell to form a pore through which effectors transit to reach the cell cytoplasm. Other substrates of the TTS apparatus are encoded by genes

scattered throughout the VP, such as *vir*A, *osp*B, C, D, E, F and G and *ipa*H genes (Gall et al., 2005). VirA induces destruction of local microtubule structures and promotes the actin-based motility of bacteria within the host cell cytoplasm. The genes encoding the *ipa*H1.4, *ipa*H2.5, *ipa*H4.5, *ipa*H7.8 and *ipa*H9.8 members of the *ipa*H gene family of *Shigella* are encoded on the 220 kb plasmid, but several *ipa*H cognate genes are also present on the chromosome. The chromosome of *Sh. flexneri* strains 301 and 2457T contains seven putative *ipa*H cognates. Their genome sequences suggest that both strains contain seven putative *ipa*H homologous genes and that three of the seven *ipa*H gene possess stop codons, thus preserving four full-length *ipa*H genes (Ashida et al., 2007).

For specific detection of *Shigella* spp. using *vir*A and *ipa*H as target genes have been reported earlier. In 1998, Villalobo and Torres described the highly sensitive and specific detection of virulent *Shigella* organisms and EIEC in mayonnaise by PCR targeted to the *vir*A. Their results showed that PCR with *vir*A primers could be a useful test and successfully apply for detection of *Shigella* and EIEC in mayonnaise. For using *ipa*H gene as target, the amplification of the invasion plasmid antigen H (*ipa*H) gene sequence is used for the diagnosis of dysentery. IpaH is carried by all four *Shigella* species (Thiem et al. 2004). In 2004, Thiem and colleagues detected *Shigella* and EIEC in rectal swab specimens from patients with diarrhea presenting by real-time PCR using *ipa*H genes as target. The *ipa*H-specific real-time PCR assay was found to be highly sensitive in relation to culture for the confirmation of shigellosis. However, the real-time PCR assay detected *ipa*H found that high number of *Shigella* culture-negative patients could be detected. They concluded that the clinical presentation of shigellosis may be directly related to the

bacterial load. In this research part, specificity and accuracy of *vir*A and *ipa*H genes were evaluated for multiple target bacteria detection.

Specific detection of *L. monocytogenes* using *prf*A gene as species specific target had been well addressed in many research. The genus *Listeria* consists of seven different species, of which only *L. monocytogenes* and *L. ivanovii* are known to be pathogenic for humans and animals. Various *Listeria* species can be distinguished from each other by their abilities to ferment specific sugars and to reduce nitrate and by differences in their hemolytic phenotypes. The pathogenesis of listerial infections is a complex process involving a number of virulence factors expressed by virulent *L. monocytogenes* (Wernars et al., 1992). Several *L. monocytogenes* virulence genes have been identified. They include genes involved in adherence and uptake by the host cell, escape from the phagocytic vacuoles and intracellular replication or intra- and intercellular movement. The majorities of these virulence determinants are located within a 10-kb chromosomal region and are regulated by the positive transcriptional regulator PrfA. PrfA is a member of the Crp/Fnr family of transcriptional activators, and members of this family appear to require posttranslational modification or the binding of a small molecule cofactor for full activity. This protein is a 27-kDa site-specific DNA-binding protein that recognizes a 14-bp palindrome (PrfA box) within the -40 region of PrfA-dependent promoters. Protein function activates all genes of the so-called virulence gene cluster of *L. monocytogenes* (*prf*A, *plc*A, *hly*, *mpl*, *act*A and *plc*B) as well as the expression of *inl*A and *inl*B, which encode two invasion proteins (InlA and InlB) (Milohanic et al., 2003; Wong and Freitag, 2004). Multiple mechanisms exist to regulate *prf*A expression and protein activity. Three promoters contribute to the transcriptional

regulation of *prf*A. The promoters *prf*Ap1 and *prf*Ap2 are located immediately upstream of the *prf*A coding region and are important for providing the initial levels of PrfA protein required for the activation of gene products essential for bacterial escape from host cell vacuoles. These promoters are functionally redundant in vivo and appear to contribute to both positive and negative regulation of *prf*A. The third promoter contributing to regulation of *prf*A is located upstream of the *plc*A gene and increases *prf*A expression via the generation of a bicistronic *plc*A-*prf*A transcript; this promoter is PrfA dependent and represents a positive feedback loop for *prf*A expression (Wong and Freitag, 2004). PrfA activation occurs upon bacterial entry into the host cell cytosol and is required for the increased expression of gene products that promote bacterial cell to-cell spread (Bruno Jr. and Freitag, 2010). The *prf*A gene, rather than individual virulence-associated genes, would be a more attractive target for use in the identification of *L. monocytogenes* if its presence could be demonstrated in all strains belonging to this pathogenic species (Wernars et al., 1992). In Chapter 3 results, oligonucleotide array targeted to *prf*A gene showed that this was specific for *L. monocytogenes* detection and no cross-reactivity with another *Listeria* species or non-target bacteria. Thus, this *prf*A gene was used as target gene for specific detection of *L. monocytogenes* using m-PCR in this part.

In this investigation, *inv*A and *fim*Y gene were evaluated for specific detection of *Salmonella* spp., *vir*A and *ipa*H were evaluated for detection of *Shigella* spp., *usp*A for detection of *E. coli* and *prf*A gene for detection of *L. monocytogenes*. Primers were designed using published data from previous research and nucleotide sequence from NCBI database (http://www.ncbi.nlm.nih.gov/).

4.1.3 Multiplex PCR methods for simultaneous detection of multiple target bacteria

For simultaneous detection of multiple target bacteria, m-PCR has been applied. M-PCR involves the simultaneous amplification of more than one target genes per reaction by mixing multiple primer pairs with different specificities. It is based on the separation of PCR amplicons of different molecular weight by agarose gel electrophoresis (Settanni and Corsetti, 2007). Simultaneous detection of different multiple target bacteria including *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp. and *L. monocytogenes* were reported earlier. In 2004, Li and Mustapha used m-PCR to detect *E. coli* O157:H7 (targeted to *uid*A gene), *Salmonella* (targeted to genus-specific sequence) and *Shigella* (targeted to *ipa*H gene) in apple cider. After 24 h enrichment, $8x10^{-1}$ CFU/ml of *E. coli* O157:H7, *Salmonella* and *Shigella* could be detected in low background bacterial samples including apple cider, cantaloupe, watermelon and tomato and 80 CFU/g of these bacteria could be detected in alfalfa. In 2005, Joffe and colleagues developed the m-PCR methods for detection of 2 target bacteria including *L. monocytogenes* and *Salmonella* spp. in cooked ham targeted to *prf*A and *inv*A genes, respectively. After 48 h enrichment in BPW, 100 CFU/g of *L. monocytogenes* and *S.* London can be detected in cooked ham. Moreover, they reported that the detection limit could be enhanced by mixing of 48 h enrichment in BPW for *Salmonella* detection and Half-Fraser broth for *L. monocytogenes* at ratio of 1:1 before DNA extraction step. By this system, 1 CFU of these bacteria could be detected in 25 g of cooked ham. In 2005, Li and colleagues applied the developed m-PCR for specific detection of *E. coli* O157:H7 (targeted to *uid*A gene), *Salmonella* spp. (targeted to genus-specific sequence), *Shigella* sp. (targeted to *ipa*H gene) to raw

and ready-to-eat meat. After 24 h enrichment in brain heart infusion broth (BHI), 2 CFU/g of *E. coli* O157:H7, *Salmonella* and *Shigella* could be detected in ground beef, roast beef, beef frankfurters, chicken nuggets, salami and turkey ham and 15 CFU/g of these bacteria could be detected in ground pork. In 2009, Germini and colleagues described the amplification of m-PCR methods for simultaneously detected *E. coli* O157:H7 (targeted to *eae*A gene), *Salmonella* spp. (targeted to *inv*A gene), *L. monocytogenes* (targeted to *prf*A gene) in eggs. Their developed method could detect 10^6 cells/ml of *E. coli* O157: H7, *Salmonella* spp. and *L. monocytogenes* in pure culture. The applications of the developed protocol for multiple foodborne pathogens in egg samples were also performed. After 15 h enrichment in TSB, 10 cells of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* could be detected in 25 g of liquid whole egg. In 2009, Kawasaki and colleagues reported the application of m-PCR for simultaneous detection of *S.* Enteritidis IF03313, *L. monocytogenes* ATCC 49594, *E. coli* O157: H7 ATCC 43894 in several kind of food such as meat, cabbage, salmon, raw egg, milk, fresh cheese, and raw ham. They showed that each pathogen was detectable by the m-PCR in all inoculated food samples with a sensitivity of 5 CFU of each pathogen per 25 g of inoculated samples after enrichment for 20 h in No17 medium. All pathogens were detected with high sensitivity in 39 inoculated samples containing natural microflora at levels ranging from <10 to 10^8 CFU/g. However, no data of detection of these bacteria prior to spike target bacteria or compared accuracy of m-PCR results with conventional culture were reported in their research.

However, all available data now, just 2 (Jofre et al., 2005) or 3 (Li and Mustapha, 2004; Li et al., 2005; Germini et al., 2009; Kawasaki et al., 2009) target bacterial species have been detected by the m-PCR method. These reports have not

combined the target genes for specific detection of *E. coli*, *Shigella* spp., *Salmonella* spp. and *L. monocytogenes* together. Moreover, a complex m-PCR with a mixture of many primers causes dramatically increasing the complexity of analysis (Wanng et al., 2007). To avoid less sensitivity of amplification of multiple target genes by m-PCR, conventional PCR were also evaluated in this research part. For conventional PCR, the efficiency of amplification is not complex and more sensitive than that of m-PCR.

However, more amplification reactions are required for simultaneous amplification of multiple genes. The detection of PCR and m-PCR amplicons is based on the separation of PCR product using different molecular weight by agarose gel electrophoresis which was less sensitive and sequencing are needed for PCR validation step (Settanni and Corsetti, 2007; Chiang et al., 2006; Wang et al., 2007). Real-time PCR were applied to improve the sensitivity of m-PCR or PCR techniques. Real-time PCR is the most commonly used technology for quantification of specific DNA fragments. The amount of product synthesized during the PCR is measured in real time by detection of the fluorescent signal produced as a result of specific amplification. However, real-time PCR requires special thermal cyclers, usually specific fluorescent probes, fluorescent detectors for detect several m-PCR product and expensive reaction reagents (Bai et al., 2010; Suo et al., 2010, Hu et al., 2011). Simultaneous detection of multiple target genes and separation of all multiple amplicons in single reaction by real-time PCR are still limited. Therefore, simple, rapid, and inexpensive methods are needed for PCR validation step for simultaneous multiple pathogens detection.

4.1.4 Combination of m-PCR and oligonucleotide array for multiple foodborne pathogen detection

The limitation of consensus gene is the cross-reactivity with some closely related bacteria. Therefore, combination of m-PCR amplification of species or genus specific genes with DNA microarray have been used for multiple pathogen detection in meat product samples (Suo et al., 2010) and clinical samples (Kim et al., 2010) using fluorescence for signal detection. In 2010, Suo and colleagues developed a low-density pathogen detection microarray using 14 species-specific genes as targets to simultaneously detect the 4 most prominent foodborne pathogens including *E. coli* O157:H7, *Salmonella enterica*, *L. monocytogenes* and *Camp. jejuni*. In the same array, the *stx*1 (encoding Shiga toxin I) and *stx*2 (encoding Shiga toxin II) probes of *E. coli* O157:H7 were also included to genotype the main virulence genes of the pathogen. Fluorescence detection systems were used for signal detection. They reported that the sensitivity of the microarray detection coupled with m-PCR amplification was at least 1×10^{-4} ng (approximately 20 copies) of the genomic DNA. The developed methods were applied to 39 raw meat samples packages including chicken, beef, pork and turkey. The aerobically growing target pathogens were concurrently enriched from meat samples in a multi-pathogen selective enrichment medium (SEL) for 20 h, and *Camp. jejuni* was enriched from chicken samples in selective Bolton broth under microaerobic condition for 48 h prior to the microarray detection. They reported that after 20 h selective enrichment in SEL, 8 packages were found to be positive for one or two of these pathogens by the microarray assay combined with 14-plex PCR. In 2010, Kim and colleagues developed and evaluated a m-PCR-oligonucleotide-based microarray system for the detection of

enteropathogenic bacteria from stool samples of patients with enteritis. They designed and tested oligonucleotide probes that targeted the virulence-factor genes of the 10 enteropathogenic bacteria that are frequently responsible for outbreaks of enteritis in Korea. Fluorescence detection systems were used for signal detection. They showed that the detection limit for genomic DNA from a single strain of *Vibrio cholera* was approximately 10 fg. In the presence of heterogeneous non-target DNA, the detection sensitivity of the array decreased to approximately 100 fg. Clinical stool samples from 82 patients with foodborne enteritis were also analyzed using the m-PCR-DNA microarray approach. The results obtained using their approach coincided with those obtained by the cultivation and PCR approaches. However, only one bacterial species were detected in each sample using their method.

For microarray detection system, fluorescent assays are commonly used methods for microarray-based detection of pathogens. Limitations of these assays are that labeling of target DNA can be inconsistent and highly variable, and they utilize expensive and nonportable scanners for data acquisition and analysis (Quiñones et al., 2011). Alternatively, colorimetric methods and biochips including optical thin-film biosensor chips (Bai et al., 2010) and ampliPHoX technology, light-initiated signal amplification through polymerization (Quiñones et al., 2011), have been developed for signal detection. Although optical thin-film biosensor chips can be detected hybridization signal by naked eyes but array chip construction need complicated preparation process for destructive interference of reflected wavelengths by using silicon nitride as the optical coating on material array. For ampliPHoX technology, detection systems need microarray construction machine, special reagents and equipment for signal detection and analysis. Other colorimetric methods are using

digoxigenin (DIG) or biotin as labeling molecule with no special equipment needed for material array construction and signal detection as review in Chapter 3. In previous reports, only conserve genes including 16S rRNA gene (Chiang et al., 2006), 23S rRNA gene (Hong et al., 2004) and *gro*EL gene (Hu et al., 2011) were selected as targets for multiple pathogen detection using (DIG) or biotin as labeling molecule. To overcome the limitation of cross-reaction in our research, consensus and genus or species specific genes were used as targets for PCR amplification prior to apply to oligonucleotide array. DIG were used for DNA labeling which no special equipment needed for material array construction and signal detection were used in this research.

4.1.5 Objectives

The aim of this research was to develop oligonucleotide array based methods for simultaneous detection of multiple foodborne pathogens in fresh chicken meat with high accuracy and easy system. The dominant target bacteria in chicken meat including *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. were chosen as the models for methods development. Moreover, *Cl. perfringens* and *Staph. aureus* which could be differentiated using only 16S rRNA gene as target were also tested in this part to evaluate the specificity and efficiency of the developed method. Oligonucleotide probes and m-PCR targeted the conserved and species or genus specific genes of the 4 target bacteria were designed and evaluated. M-PCR products were distinguished from each other in the oligonucleotide array by post-PCR labeling using DIG.

Alternatively, to avoid the problems of false negative from m-PCR amplification, the convention PCR were also used to separately amplify each target

gene of interest using the same PCR amplification condition. The amplicon products were mixed together and distinguished from each other in a single array. The application of the assay to fresh chicken meat samples was addressed as well.

4.2 Materials and methods

4.2.1 Bacterial strains and cultivation

All bacterial reference and isolated strains used to validate oligonucleotide array probes are listed in Table 4.1. All isolated strains of target and non-target bacteria used in this study were identified using biochemical characteristic profiles as described by United States Food and Drug Administration – Bacteriological Analytical Manual (United States Food and Drug Administration, 1998). All target bacteria except for *Cl. perfringens* were grown on TSA (Appendix I, M1.6). For the cultivation of *Cl. perfringens*, the bacterium was cultured on TSC (Biomark) and incubated under anaerobic condition at 37^{o}C for 24 h.

Table 4.1 Bacterial strains used for the validation of oligonucleotide array and m-PCR

Species	**Number of strains**	**Strain number and sources**
E. coli	7	*E. coli* TISTR[a] 887, *E. coli* E[b] 1, 2, 3, 4, 6, 7
Cl. perfringens	1	*Cl. perfringens* CP[b]5
L. monocytogenes	8	*L. monocytogenes* DSM[a] 12464, DMST[a] 1327, 2871, 20093, 21164, 23136, 23145, 31802
Salmonella spp.	8	*S.* Enteritidis JCM 1652, TISTR 2394, *S.* Typhimurium TISTR 292, *Salmonella* sp. S[b]2, 3, BC[b]1, L6, CM[b]7

Table 4.1 (Continued)

Species	Number of strains	Strain number and sources
Shigella spp.	12	*Shigella boydii* DMST 3395, 28180, 30245, *Sh. dysenteriae* DMST 2137, 5875, 15111, *Sh. flexneri* DMST 17559, 17560, 30581, *Sh. sonnei* DMST 17561, 23595, *Shigella* sp. Sh[c]1
Staph. aureus	1	*Staph. aureus* TISTR 517
Non-target bacteria found in enrichment culture	16	C[b]2, 3, 4, 6, 7, RV[b]2, 3, TT[b]1, 2, 3, 9 L[b]2, 4, 5, 7, 8

[a] Reference strain: DMST, The Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH German Collection of Microorganisms and Cell Cultures; JCM, Japan Collection of Microorganisms; TISTR ,Thailand Institute of Scientific and Technology Research.

[b] Strains isolated from chicken intestine in Nakhon Ratchasima, Thailand: BC, *Salmonella* sp. enriched using RV broth and isolated on XLD agar; C, non-*E. coli* bacteria isolated on EMB agar; CM, *Salmonella* sp. isolated on mCCDA; CP, *Cl. perfringens*; E: *E. coli*; L, non-*Listeria* bacteria isolated on PALCAM agar; RV, non-*Salmonella* bacteria enriched using RV broth and isolated on XLD agar; S: *Salmonella* sp. enriched using TT broth and isolated on XLD agar; TT, non-*Salmonella* bacteria enriched using TT broth and isolated on XLD agar

[c] Strains isolated from food in Khon Kaen, Thailand: CP, *Cl. perfringens*; Sh, *Shigella* sp.

4.2.2 Primer and probe design

For the conserved gene, the 16S rRNA genes specific for each bacterial species and genus were also used as target regions. Universal 16S rDNA primers as used in Chapter 3 are shown in Table 4.2. For genus or species specific genes, primers for amplification of the specific genes were designed based on the conserved regions

of each gene in each target bacteria. The genus specific gene were *fim*Y for specific detection of *Salmonella* spp., *ipa*H for detection of *Shigella* spp. Species specific gene were *usp*A (Chen and Griffen, 1998) and *prf*A for specific detection of *E. coli* and *L. monocytogenes*, respectively. Moreover, some published primer targeted to the specific genes including *inv*A for specific detection of *Salmonella* spp. and *vir*A for specific detection of *Shigella* spp. (Mao et al., 2008) were also validated to obtain suitable target gene for detection of local isolated strains in Thailand.

Specificity of each primer was primarily tested using Primer-Blast program in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of all forward and reverse primers are shown in Table 4.2. All primers were also tested for specificity with genomic DNA extracted from reference and isolated bacterial strains in Table 4.1. Probe specific for each pathogen for oligonucleotide array were designed based on the variable regions of the 16S rDNA and conserve regions of each target genes using the PICKY oligonucleotide design program (Chou et al., 2004). Sequences of each probe are shown in Table 4.3.

Table 4.2 Primers used for target gene amplifications by conventional PCR and m-PCR

Species	Target gene	Primer sequences (5' to 3')	PCR product size (bp)	References
All species	16S rRNA	**F**: AGACTCCTACGGGAGGC **R:** GGTAAGGTTCTTCGCGT	625-655	This work
E. coli	*usp*A	**F:** CCGATACGCTGCCAATCAGT **R:** ACGCAGACCGTAGGCCAGAT	884	Chen and Griffiths (1998)

Table 4.2 (Continued)

Species	Target gene	Primer sequences (5' to 3')	PCR product size (bp)	References
L. monocytogenes	*prf*A	**F**: CACAAGAATATTGTATTTTTCTATATGAT **R**: CAGTGTAATCTTGATGCCATCA	398	This work
Salmonella spp.	*fim*Y	**F**: CGGCTAAAGCTTTCCGATAAGCG **R**: AAATGCTAAAGACTGCGCCTGCCG	489	This work
Salmonella spp.	*inv*A	**F**: GAAATTATCGCCACGTTCGGGCAA **R**: TCATCGCACCGTCAAAGGAACC	283	Mao et al. (2008)
Shigella spp.	*ipa*H	**F:** GAGGACATTGCCCGGGATAAAG **R:** TAAATCTGCTGTTCAGTCTCACGC	422	This work
Shigella spp.	*vir*A	**F:** CTGCATTCTGGCAATCTCTTCACATC **R:** TGATGAGCTAACTTCGTAAGCCCTCC	215	Mao et al. (2008)

4.2.3 Target gene amplification by m-PCR or conventional PCR based technique

The genomic DNA templates for target genes amplification were prepared from 16-24 h grown pure cultures on TSA (Appendix I, M1.6) or TSC (Biomark) using the simple protocol of phenol-chloroform based method as described in Chapter 3 (3.2.3).

For amplification of each target gene, the PCR reactions were performed individually in a total volume of 25 µl containing 1× GoTaq Flexi buffer (Promega, Madison, WI USA), 1 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.4 µM forward and reverse primers (Table 4.2), 0.5 U GoTaq Flexi DNA polymerase (Promega), and 100

ng DNA templates. The amplification for each target gene was done separately for each template. The PCR reactions were heated at 95^{o}C for 3 min and then, 35 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 60 s followed by a final step of 5 min incubation at 72°C. The PCR products were analyzed by electrophoresis on 1% agarose gel.

For m-PCR amplification, all PCR components in the m-PCR were performed as described in the amplification of each specific gene, except for the amplification conditions and primer concentrations were optimized. In all m-PCR reactions, 16S rRNA gene was used as an internal control. The concentration of each gene specific primer and annealing temperature were varied for the m-PCR reaction. The optimum condition of m-PCR was the condition which all target bacteria can be amplified using the lowest concentration of DNA template with no cross amplification with the non-target organisms. The products of m-PCR were analyzed by electrophoresis on 4% agarose gel and purified using QIAquick PCR Purification Kit (Qiagen, GmbH, Hilden Germany). The concentration of genomic DNA template and PCR product were measured by Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

4.2.4 Oligonucleotide array preparation and detection

Nylon membranes (Roche, Mannheim, Germany) were used as the array matrix. Single strand probes (Table 4.3) were heated at 95^{o}C for 5 min, and 200 pmol were spotted on a specific position on the dry nylon membrane (Figure 4.4A). The membranes spotted with probes were exposed to UV for 3 min to allow cross-linking.

For oligonucleotide array probes validation and selection, m-PCR containing all suitable primers pairs were used for target gene amplification from

several bacterial strains. Two hundred ng of purified m-PCR products were denatured at 99^{o}C for 10 min and quickly chilled on ice. The denatured DNA were labeled with 2 µl of DIG High Prime (Roche) and incubated at 37^{o}C for 1 h. Membranes with spotted probes were pre-hybridized in a pre-warmed DIG Easy Hybridization solution (Roche) at 35^{o}C with gentle shaking for 30 min. Prior to hybridization, 10 µl of labeled PCR products reactions were heated to 99^{o}C for 5 min, then immediately cooled on ice and added to 2 ml of newly pre-warmed hybridization solution. The hybridizations were carried out at 35^{o}C with gentle rotating for 4 h. After hybridization, the membranes were washed as described in Chapter 3 (3.2.4). Then the membranes were incubated for 30 min in blocking solution (Roche) and 30 min in antibody solution (Roche). After washing twice in washing solution (Roche) for 15 min each, the membranes were equilibrated in detection buffer (Roche) for 2 min, and in freshly prepared NBT/BCIP (Roche) color substrate solution in the dark for 4-8 h. The results were visualized and photographed.

4.2.5 Sensitivity detection assay

To determine the sensitivity of the oligonucleotide array for detecting bacteria, *S.* Enteritidis JCM 1652, *E. coli* TISTR 887, *Sh. boydii* DMST 28180 and *L. monocytogenes* DSM 12464 were separately grown in TSB at 37°C for 18–24 h. A 10-fold dilution series of each bacterial culture were prepared using 0.85% sodium chloride solution. The bacterial genomic DNA was extracted from the 1 ml cell dilution. At the same time, enumeration was done by spreading 100 µl dilutions onto TSA (Appendix I, M1.6) plates for viable count. The efficiency and sensitivity of the m-PCR or conventional PCR combined with oligonucleotide array for simultaneous detection of the multiple target bacteria was evaluated. A genomic DNA mixture

consisting of the same volume of genomic DNA extracted from each target bacteria (concentration for each at 10^6-10^0 cells or 10 – 0.001 ng/ µl) was used as templates for amplification of each target genes. One µl of mixed genomic DNA at different cell or genomic DNA concentration were amplified in single reaction with several gene specific primers using m-PCR or amplified individually with each gene specific primer using conventional PCR.

For m-PCR, 10 ul of m-PCR products obtained from amplification of each concentration of genomic DNA mixture were labeled with 2 µl of DIG High Prime (Roche) prior to apply to oligonucleotide array. For conventional PCR, the amplified products of each target gene were mixed at the same volume. Ten ul of mixed PCR product were labeled with 2 µl DIG High Prime (Roche) and then applied to oligonucleotide array assay.

4.2.6 Application of oligonucleotide array for multiple foodborne pathogen detection in fresh chicken meat

Total of 4 fresh chicken meat including 2 breasts, 1 thigh, and 1 wing were collected from open market and supermarket in local area. All 4 samples were spiked with target bacteria at different final concentration. Twenty-five grams of each meat sample was placed in a stomacher bag and spiked with 100 µl of each cells dilution including *L. monocytogenes* DSM 12464, *S.* Enteritidis JCM 1652, and *Sh. boydii* DMST 28180 at final concentration of 1-200 cells. All spiked and non-spiked of each sample were added with 225 ml of pre-enrichment medium including BPW (Appendix I, M1.1) for *Salmonella* and *E. coli* enrichment or Half Fraser broth (HF) (OXIOD, Basingstoke, United Kingdom) for *L. monocytogenes* enrichment or Shigella broth (Appendix I, M1.5) for *Sh. boydii* enrichment. Then, samples were

homogenized at normal speed for 1 min using laboratory blender stomacher 400 (Seward Laboratory System Inc., New York, USA). All homogenized mixtures were incubated for 24 h at 37^{o}C under aerobic condition for *Salmonella*, *E. coli* enrichment, under anaerobic condition at 42^{o}C for *Shigella* enrichment and at room temperature for *L. monocytogenes* pre-enrichment. One ml of BPW cultures were transferred to 10 ml Rappaport-Vassiliadis broth (RV) (Himedia, Mumbai, India) and 10 ml tetrathionate broth (TT) (Himedia) and incubated at 42^{o}C for 24 h for *Salmonella* detection. For detection of *L. monocytogenes*, 100 µl of HF cultures were transferred to 10 ml Fraser broth (OXIOD) and incubated at 37^{o}C for 24 h. After 24 or 48 h incubation, aliquots of each enrichment culture from each sample were subjected to the oligonucleotide array and conventional analyses.

In conventional analyses, for *Sh. boydii* detection, the cultures of Shigella broth were streaked on MacConkey agar (Himedia, Mumbai, India). For *E. coli* detection, the BPW cultures and Shigella broth (Appendix I, M1.5) were streaked on Eosin-Methylene Blue agar (EMB) (Himedia). For *Salmonella* detection, the cultures of RV and TT broth were streaked on xylose lysine desoxycholate (XLD) agar (OXIOD) and on bismuth sulfite (BS) agar (OXIOD). *L. monocytogenes* was detected by streaking of Fraser broth culture on PALCAM agar (OXIOD). The incubation of the target bacteria on selective agars were incubated at 37^{o}C for 24 h for *E. coli*, *Shigella*, and *Salmonella* detection and for 48 h for *L. monocytogenes* detection. The suspected colonies of each target bacterium on selective agar were re-streaked. Single colonies were picked and mixed in 20 µl of water, heated at 100^{o}C for 10 min and 1 µl of supernatant was used directly as the template in the m-PCR reaction for bacterial colony confirmation.

For oligonucleotide array assay, 1 ml of BPW, RV, TT, Shigella, and Fraser broth culture were separately collected. Cell pellets were harvested by centrifugation and washed once in 0.85% sodium chloride solution and extracted for genomic DNA using phenol-chloroform based method (Kumar et al., 2008) as described in Chapter 3. The total genomic DNA pellet was dissolved in 50 µl TE, pH8.

For simultaneous detection of multiple target bacteria by m-PCR-oligonucleotide array, an equal volume of total genomic DNA solution obtained from each enrichment culture was mixed and 1 µl of genomic DNA mixture were used as template for m-PCR amplification. For *L. monocytogenes* detection, 1 µl of genomic DNA extracted from Fraser culture was used as templates only. Ten µl of the m-PCR products from mixed enrichment culture and Fraser culture were individually labeled with 2 µl of DIG High Prime (Roche) and apply to oligonucleotide array separately. Hybridization patterns of both arrays were combined for 4 target bacteria detection for each sample.

For simultaneous detection of multiple target bacteria by conventional PCR-oligonucleotide array, an equal volume of total genomic DNA solution obtained from each enrichment culture (BPW, RV, TT, Shigella, and Fraser broth) was mixed and 1 µl of genomic DNA mixture were used as template for separately amplification of 16S rRNA, *fim*Y, *ipa*H, *usp*A, specific gene. For *prf*A gene amplification, total genomic DNA solution obtained from Fraser culture was used as template alone. An equal volume of each amplified products were mixed. Ten µl of mixed PCR products were labeled with 2 µl of DIG High Prime (Roche) and apply to a single oligonucleotide array.

4.3 Results

4.3.1 Optimization and specificity of the m-PCR

The specificities of *fim*Y, *inv*A, *ipa*H, *prf*A, *vir*A, and *usp*A genes (Table 4.2) were tested with genomic DNA templates extracted from pure cultures of *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. Based on the specificity and ability of amplification in m-PCR, the suitable target genes were *fim*Y, *ipa*H, *prf*A, and *usp*A. The concentrations of gene specific primers for *ipa*H, *fim*Y, *prf*A, *usp*A, and 16S rRNA gene amplification were varied from 0.02 – 0.4 µM. The annealing temperatures of the m-PCR reactions were varied from 50-59°C. The optimum concentrations of the primers set for amplification of target bacteria by m-PCR reaction were 0.02 µM *ipa*H, 0.036 µM *fim*Y, 0.06 µM *usp*A, 0.12 µM *prf*A and 0.4 µM 16S rRNA (internal control). The optimum annealing temperature for the m-PCR was 52^{o}C. The expected PCR products of 884, 489, 422 and 398 bp were found from specific amplification of both reference and isolated strains of *E. coli*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*, respectively (Figure 4.1). The 650 bp 16S rRNA gene internal control amplification can be observed from all bacterial amplification. However, the amplification of *usp*A gene was also detected from all 4 *Shigella* species but *Shigella* can be differentiated from *E. coli* by the present of *ipa*H gene product (Figure 4.1B, Lanes 1-11). These results demonstrated that the specific detection of *E. coli*, *Salmonella* spp., *L. monocytogenes*, and *Shigella* spp. can be done using m-PCR developed from this investigation.

(A)

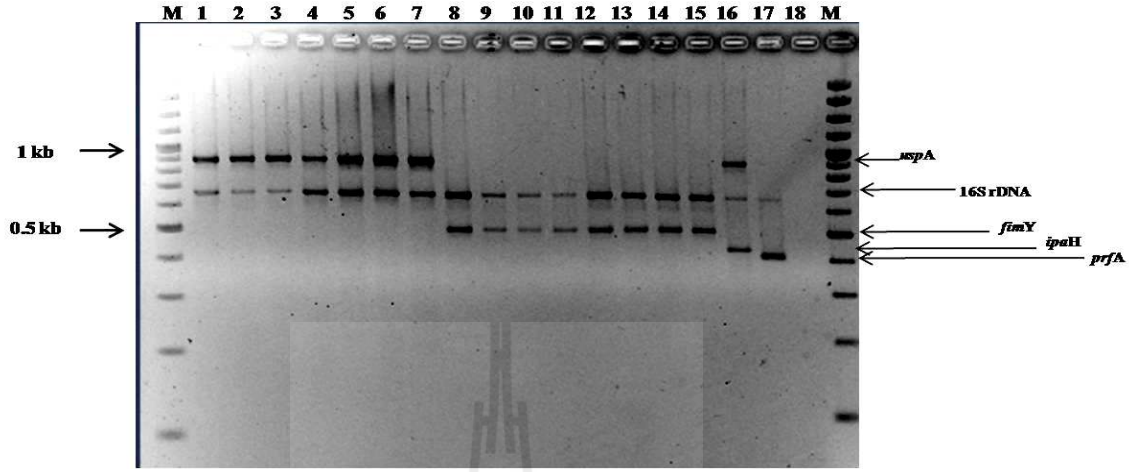


Figure 4.1 Primer validations for specific detection of target bacteria using m-PCR technique**. (A)** Specific detection of *E. coli* and *Salmonella* spp. using m-PCR technique. Lanes: 1-7, *E. coli* isolates E1, 2, 3, 4, 6, 7, TISTR 887, respectively; 8-15, *Salmonella* sp. isolates S2, 3, BC1, L6, CM7, *S.* Enteritidis JCM 1652, TISTR 2394, *S.* Typhimurium TISTR 292, respectively; 16, *Shigella* sp. isolate Sh1; 17, *L. monocytogenes* DSM 12464; 18, negative control (H_2O)**;** M, 100 bp DNA marker (Fermentas).

(B)

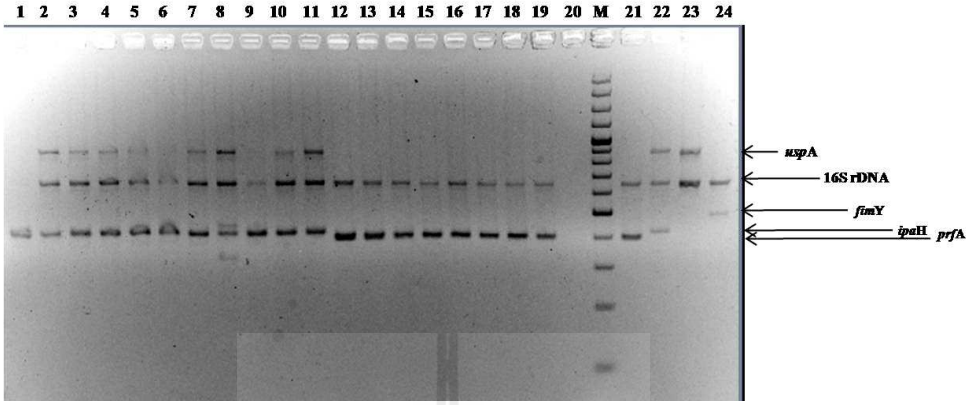


Figure 4.1 Primer validations for specific detection of target bacteria using m-PCR technique. **(B)** Specific detection of *Shigella* spp. and *L. monocytogenes* using m-PCR technique. Lanes: 1-3, *Shigella boydii* DMST 3395, 28180, 30245, respectively; 4-6, *Sh. dysenteriae* DMST 2137, 5875, 15111, respectively; 7-9, *Sh. flexneri* DMST 17559, 17560, 30581, respectively; 10-11, *Sh. sonnei* DMST 17561, 23595, respectively; 12-19, *L. monocytogenes* DMST 1327, 2871, 17303, 20093, 21164, 23136, 23145, 31802, respectively; 20, negative control (H_2O); 21, *L. monocytogenes* DSM 12464; 22, *Shigella* sp. isolate Sh1; 23, *E. coli* TISTR 887; 24, *S.* Enteritidis JCM 1652; M, 100 bp DNA marker (Fermentas).

The m-PCR specificity was tested using non-target bacteria isolated from each enrichment culture (Table 4.1). Only the 16S rDNA gene product was detected from the non-target bacteria (Figure 4.2). These results demonstrated that the target genes reported here can be used for specific detection of only target bacteria. Thus

these foodborne pathogens could be directly detected from the enrichment culture with high accuracy and no cross-reactivity with other non-target bacteria. The m-PCR developed in this study also tested for efficiency for multiple pathogen detection in a single reaction using genomic DNA mixture consisting of 4 target bacteria as templates. The results indicated that each target bacteria in mixed genomic DNA template could be detected in a single amplification reaction as the expected PCR products on 4% agarose gel were seen (Figure 4.3).

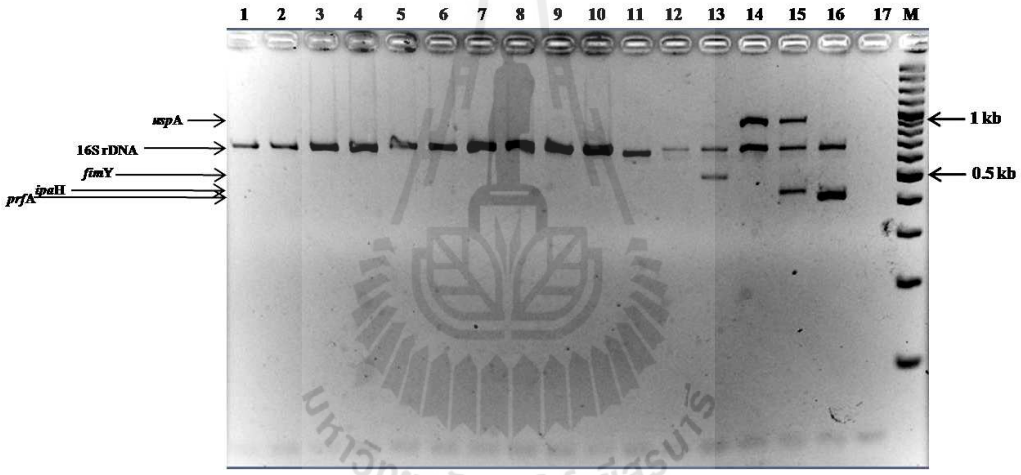


Figure 4.2 Specificity of m-PCR amplification using genomic DNA of target and non-target bacteria as templates. Lanes: 1-4, non-*E. coli* isolates C2, 3, 4, 6, respectively; 5-7, non-*Salmonella* isolates RV2, RV3, TT1, respectively; 8-10, non-*Listeria* isolates L2, 4, 5, respectively; 11, *Cl. perfringens* isolate CP5; 12-17: *Staph. aureus* TISTR 517, *S.* Enteritidis JCM 1652, *E. coli* TISTR 887, *Shigella* sp. isolate Sh1, *L. monocytogenes* DSM 12464, negative control (H_2O), respectively; M, 100 bp DNA marker (Fermentas).

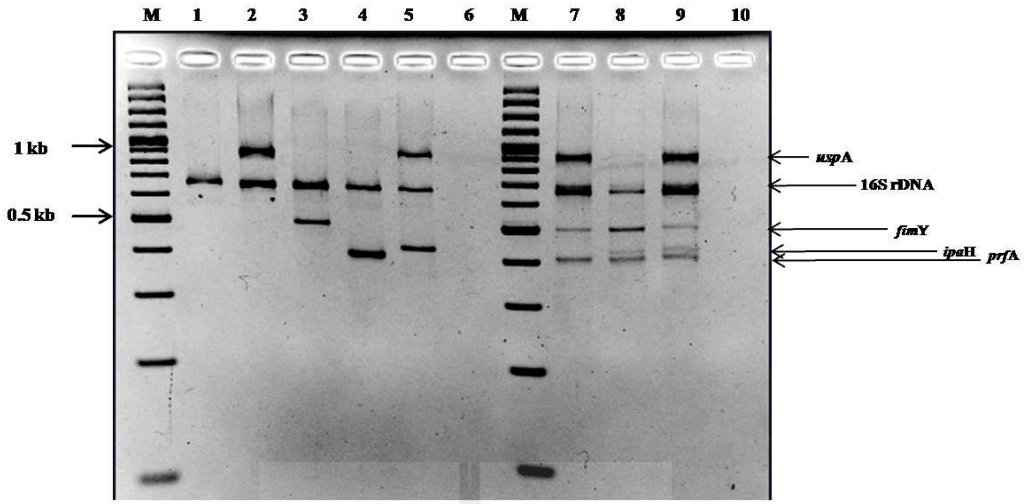


Figure 4.3 Multiple target pathogen detection by m-PCR. Lanes: 1-6, m-PCR products amplification from *Staph. aureus* TISTR 517, *E. coli* TISTR 887, *S.* Enteritidis JCM 1652, *L. monocytogenes* DSM 12464, *Shigella* sp. isolate Sh1, negative control, respectively; 7-9, m-PCR products amplification from mixed templates of *E. coli*, *L. monocytogenes*, *S.* Enteritidis; mixed templates of *L. monocytogenes*, *S.* Enteritidis, *Shigella* sp. isolate Sh1; mixed templates of *E. coli*, *L. monocytogenes*, *S.* Enteritidis, *Shigella* sp. isolate Sh1; 10, negative control (H_2O), respectively; M, 100 bp DNA marker (Fermentas).

4.3.2 Probe validation and specificity test

To detect multiple target bacteria using combination of m-PCR and oligonucleotide array, 2-7 oligonucleotide array probes against each target gene were designed. Results of probe design are shown in Table 4.3. The probes would bind within the amplified PCR fragments.

Table 4.3 Sequences of the 16S rDNA and gene specific probes spotted on the oligonucleotide array

Species	Probe name and sequences (5' to 3')	Target gene	References
Cl. perfringens	CP 1: AAGCTCTGTCTTTGGGGAAGATAATGACGG	16S rRNA	This work
	CP 3: TCCAAACTGGTTATCTAGAGTGCA		This work
	CP 4: GGCGGATGATTAAGTGGGATGT		Mao et al. (2008), This work
E. coli	EC 1: AGGAAGGGAGTAAAGTTAATACCTTTGCT	16S rRNA	Chiang et al. (2006) , Mao et al. (2008), This work
	EC 2: CTGCATCTGATACTGGCAAG		This work
Salmonella spp.	SM 1: AGGAAGGTGTTGTGGTTAATAAC	16S rRNA	This work
	SM 2: TCTGTCAAGTCGGATGTGAA		Chiang et al. (2006), This work
Staph. aureus	SA 1: AGAACATATGTGTAAGTAACTGTGC	16S rRNA	Mao et al. (2008), This work
	SA 2: CGCAGAGATATGGAGGAACA		This work
Listeria spp.	LM 1: GCTTGTCCCTTGACGGTATCTAACC	16S rRNA	This work
	LM 2: GTTTTCGGATCGTAAAGTACTGTTGTTAGAGA		Mao et al. (2008)
Campylobacter spp.	CJ 1: AGGCAGATGGAATTGGTGGTGTAGG	16S rRNA	This work
	CJ 2: AGCGTAAACTCCTTTTCTTAGGGA		This work
E. coli	UA 1: AAGAGACACATCATGCGCTGACCGAGCT	*usp*A	This work
	UA 2: GGTAGAGAAAGCAGTCTCTATGGCTCGCCC		This work
	UA 3: ACCGTTCACGTTGATATGCTGATTGTTCCG		This work
	UA 4: TTGTTTATCTAACGAGTAAGCAAG		This work
	UA 5: AAGGTAAGGATGGTCTTAACACTGAAT		This work
	UA 6: GGTGACGTAACGGCACAAGAAACGCTAGCT		This work
L. monocytogenes	PA 1: ATCCTGACCTATGTGTCTATGGTAAAGAA	*prf*A	This work
	PA 2: ACGGGAAGCTTGGCTCTATTTTGCGG		This work
	PA 3: AGCTTACAAGTATTAGCGAGAACGGGACCA		This work
	PA 4: ACAAAGGTGCTTTCGTTATAATGTCTGGCT		This work
	PA 5: AATTTAGAAGTCATTAGCGAACAGGCT		This work
	PA 6: CATACAGCCTAGCTAAATTTAATGAT		This work
	PA 7: AAACATCGGTTGGCTATTATAAGTTTAG		This work

Table 4.3 (Continued)

Species	Probe name and sequences (5' to 3')	Target gene	References
Salmonella spp.	FY 1: GCCTCAATACAGGAGACAGGTAGCGCC	*fim*Y	This work
	FY 2: ATATCGCTTTGTTGCCAACTGAGCGC		This work
	FY 3: AAATAAGTAGTGACTCAATGAATAGCCGAG		This work
	FY 4: AGTTGTAATTATTGCCTGAGAAATGATAC		This work
Shigella spp.	IH 1: GGGAGTGACAGCAAATGACCTCCGC	*ipa*H	This work
	IH 2: CGGCACTGGTTCTCCCTCTGGGGACCA		This work
	IH 3: TGTGGATGAGATAGAAGTCTACCTGG		This work
	IH 4: AGAATGAGTACTCTCAGAGGGTGGCTGAC		This work
	IH 5: AGAAACTTCAGCTCTCCACTGCCGTGA		This work

In this investigation, the DNAs amplified from 53 bacterial strains including reference and isolated strains of *E. coli*, *Salmonella*, *Shigella*, *L. monocytogenes*, *Staph. aureus*, *Cl. perfringens* and non-target bacteria isolated from enrichment culture (Table 4.1) were employed to evaluate the performance of the assay. Validation and accuracy of these probes were tested using m-PCR for amplification. The accuracy of each probe is summarized in Table 4.4. Weak signals were observed from hybridization of m-PCR product of *E. coli* with probe UA 4, and *L. monocytogenes* with probes PA 1 and PA 6. Therefore, these 3 probes were removed from the array and not determined for the accuracy and cross-reactivity with other bacteria. After hybridization, hybridization signals on the array were unambiguously distinguished for each of the species as shown in Figure 4.4B-E.

Table 4.4 Probe validation and specificity test using isolated and reference strains of target and non-target bacteria

Target bacteria	Target gene	Probe name	Number of target bacterial strains (T)		Number of non-target bacterial strains (NT)		% Accuracy [b]
			Total number of tested strains	Positive signal detection (T^+)	Total number of tested stains	Positive signal detection (NT^+)	
E. coli	*usp*A	UA 1	7	7	46	12	73.9
		UA 2	7	7	46	12	73.9
		UA 3	7	7	46	12	73.9
		UA 4	1	0	ND[a]	ND	ND
		UA 5	7	7	46	12	73.9
		UA 6	7	7	46	12	73.9
L. monocytogenes	*prf*A	PA 1	1	0	ND	ND	ND
		PA 2	8	8	45	0	100
		PA 3	8	8	45	0	100
		PA 4	8	8	45	0	100
		PA 5	8	8	45	0	100
		PA 6	1	0	ND	ND	ND
		PA 7	8	8	45	0	100
Salmonella spp.	*fim*Y	FY 1	8	8	45	0	100
		FY 2	8	8	45	0	100
		FY 3	8	4	45	0	50
		FY 4	8	8	45	0	100
Shigella spp.	*ipa*H	IH 1	12	12	41	0	100
		IH 2	12	12	41	0	100
		IH 3	12	12	41	0	100
		IH 4	12	12	41	0	100
		IH 5	12	12	41	0	100

[a] ND: Not Determine

[b] % Accuracy = $[(T^+ \times 100)/ \text{No. T}] - [(NT^+ \times 100)/ \text{No. NT}]$

(A)

SM 1	SM 2	FY 1		CJ 1	CJ 2		SA 1	SA 2
FY 2	FY 3	FY 4		CP 1	CP 3	CP 4		
	P		16S				EC 1	EC 2
				UA 1	UA 2	UA 3	UA 5	UA 6
LM 1	LM 2			IH 1	IH 2	IH 3	IH 4	IH 5
PA 2	PA 3	PA 4	PA 5	PA 7				

(B)

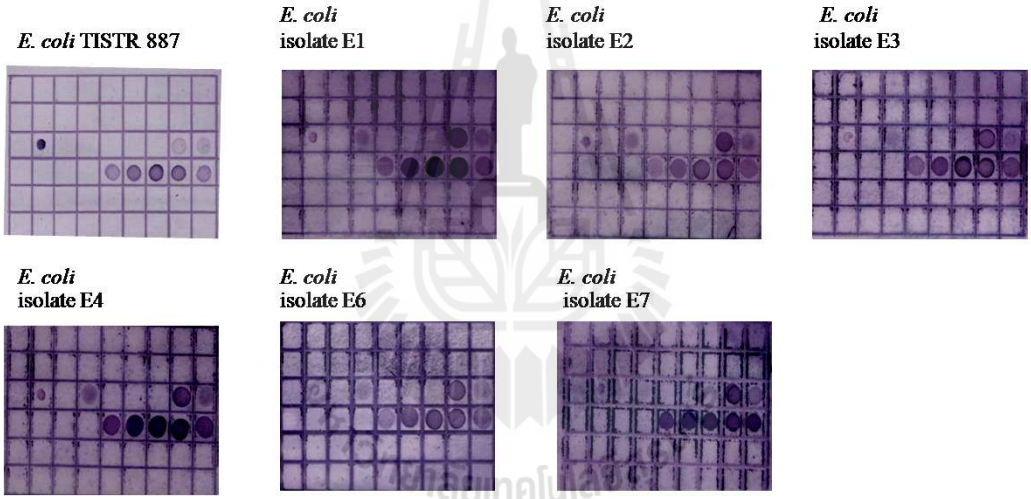


Figure 4.4 Probe validation and specific hybridization patterns of target bacteria. **(A)** Position of specific probes on nylon membrane**.** Positive controls are 0.1 ng of DIG-labeled control DNA (pBR328 DNA, linearized with *Bam* HI) (P) and 200 pmol 16S rDNA forward primer (16S). The abbreviated letters in grids are probe names as shown in Table 4.3. **(B)** Specific hybridization of individual m-PCR amplification products from reference and isolated strains of *E. coli* with specific probes on array.

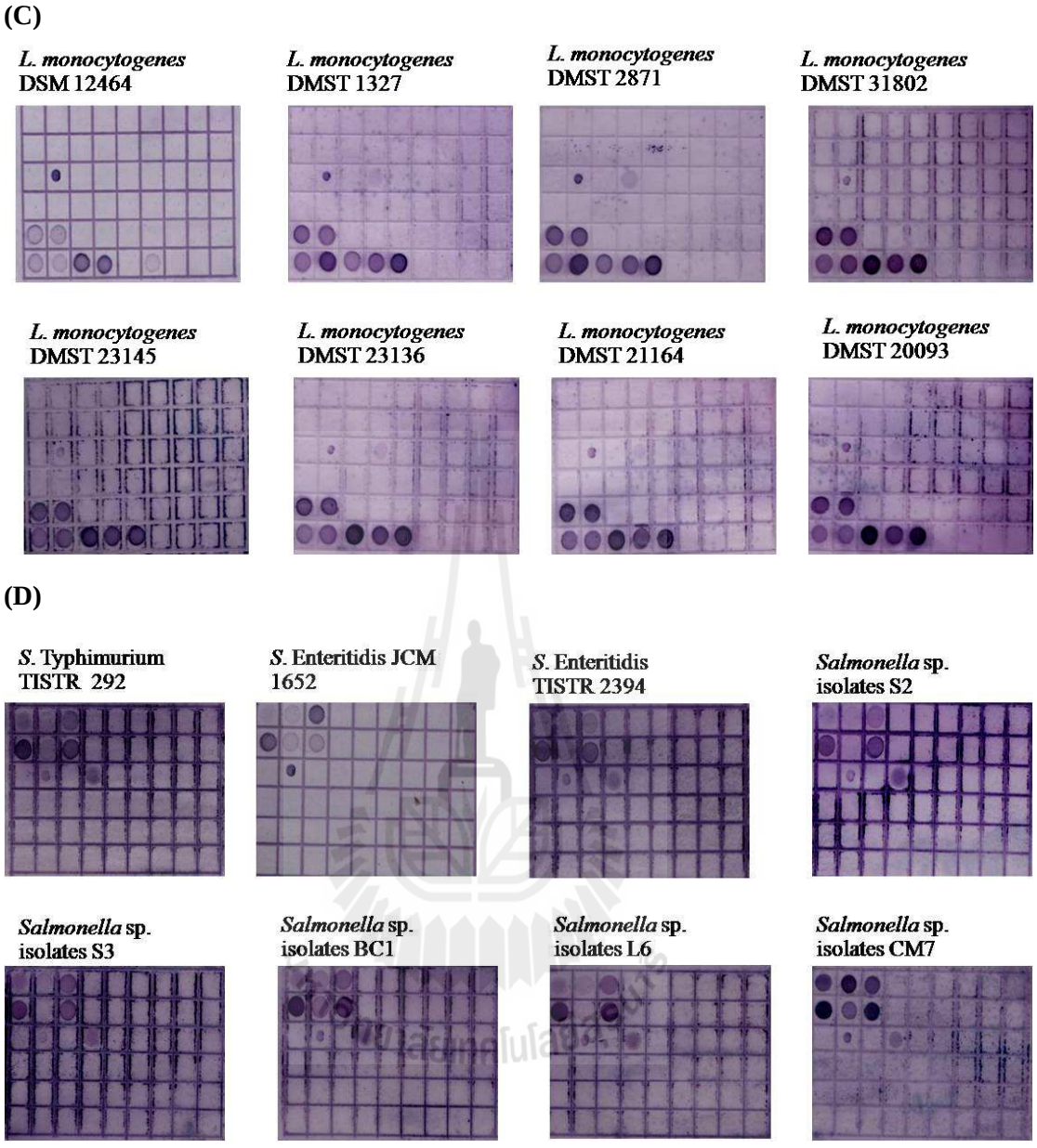


Figure 4.4 Probe validation and specific hybridization patterns of target bacteria. Position of specific probes on nylon membrane is shown in Figure 4.4A. **(C)** Specific hybridization of individual m-PCR amplification products from reference and isolated strains of *L. monocytogenes* with specific probes on array. **(D)** Specific hybridization of individual m-PCR amplification products from reference and isolated strains of *Salmonella* with specific probes on array.

(E)

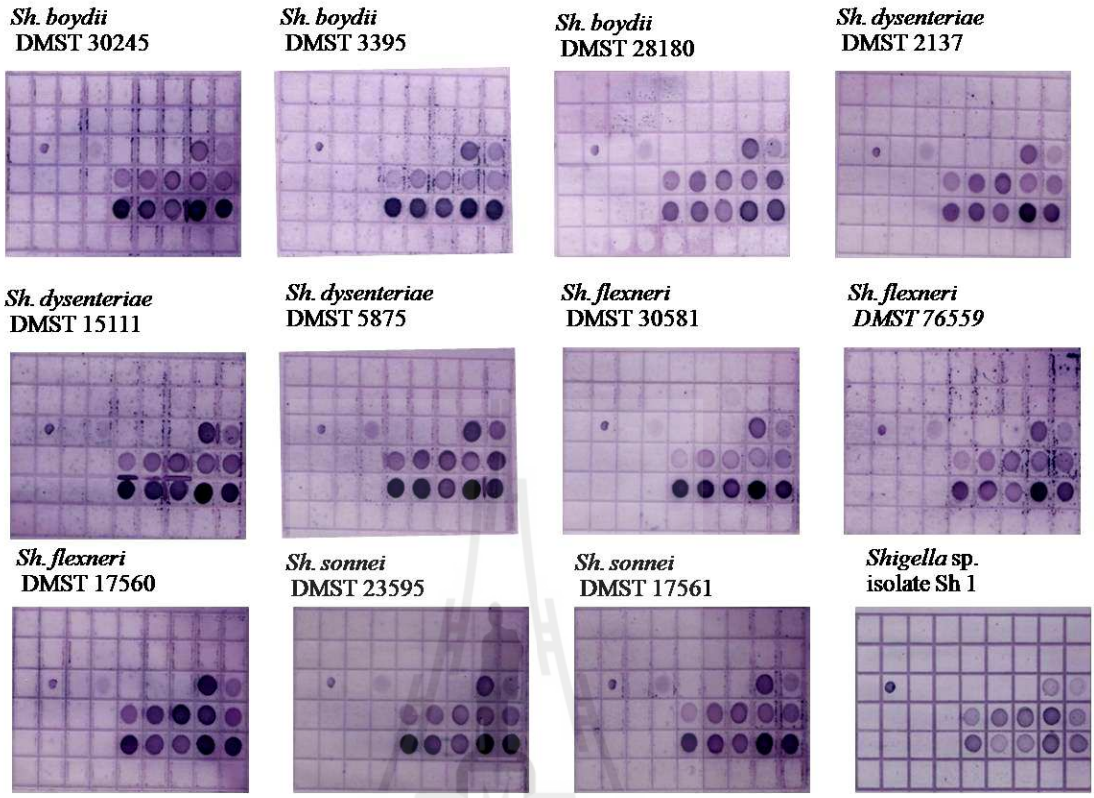


Figure 4.4 Probe validation and specific hybridization patterns of target bacteria. Position of specific probes on nylon membrane is shown in Figure 4.4A. **(E)** Specific hybridization of individual m-PCR amplification products from reference and isolated strains of *Shigella* with specific probes on array.

No cross hybridizations were observed from m-PCR product of each target bacteria except *usp*A gene of *E. coli* which can be amplified from all 4 *Shigella* species (Figure 4.4B and E). The UA probes (UA 1, 2, 3, 5, 6) targeted to *usp*A gene of *E. coli* showed only 73% accuracy (Table 4.4) because positive signals could be found from 4 species of *Shigella* (12 strains) (Figure 4.4E). However, *Shigella* can be differentiated from *E. coli* by the presence of the *ipa*H gene product.

The specificity of m-PCR combined with oligonucleotide array was also tested using non-target bacteria isolated from chicken intestine and grown in each enrichment culture (Table 4.1). Only cross-reaction of these m-PCR products with probes *E. coli* (EC) and *Salmonella* spp. (SM) targeted to 16S rRNA gene were observed (Figure 4.5). No cross-reactivity between m-PCR products of non-target bacteria in enrichment culture and gene specific probes on array were detected (Figure 4.5). These results demonstrated that probes targeted to specific genes reported here can be used for specific detection of *E. coli*, *Salmonella* spp., *L. monocytogenes*, and *Shigella* spp. by combination of m-PCR and oligonucleotide array developed from this investigation.

For multiple target bacteria detection, mixture of genomic DNA (2 ng of each genomic DNA) from each target bacteria were used as templates for multiple target bacterial detection by the m-PCR follow by oligonucleotide array. Two hundred ng of total purified m-PCR products were hybridized with specific probes on a single array. Results indicated that the hybridization patterns were found to be accurate and specific hybridization patterns of each target bacteria were seen (Figure 4.6). These results demonstrated that the developed oligonucleotide array could enhance the accuracy and simplicity of the resultant interpretation of the m-PCR detection. Taken together, these results demonstrated the ability of the oligonucleotide array combined with m-PCR amplification, to specifically identify the 4 and 5 target bacteria.

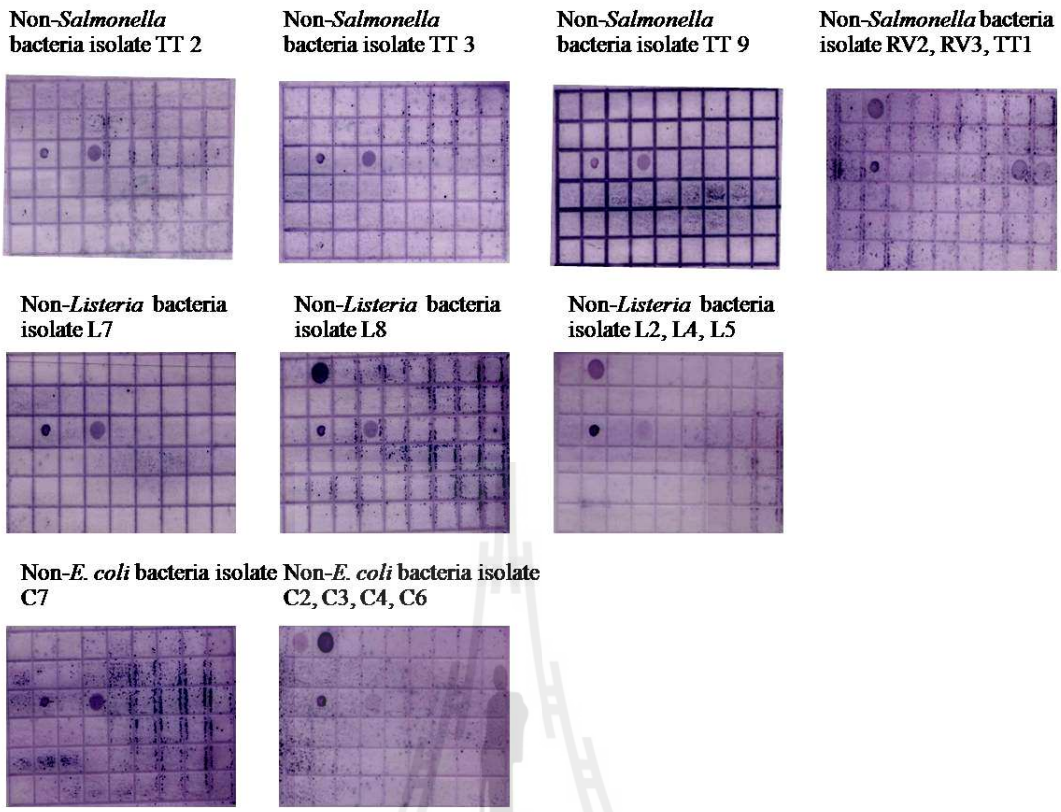


Figure 4.5 Specificity of combination of m-PCR and oligonucleotide array using genomic DNA of non-target bacteria isolated from enrichment culture as templates for analysis. Positions of specific probes on nylon membrane are shown in Figure 4.4A. The target gene for m-PCR amplification were 16S rRNA and 4 specific genes including *fim*Y, *ipa*H, *prf*A, and *usp*A.

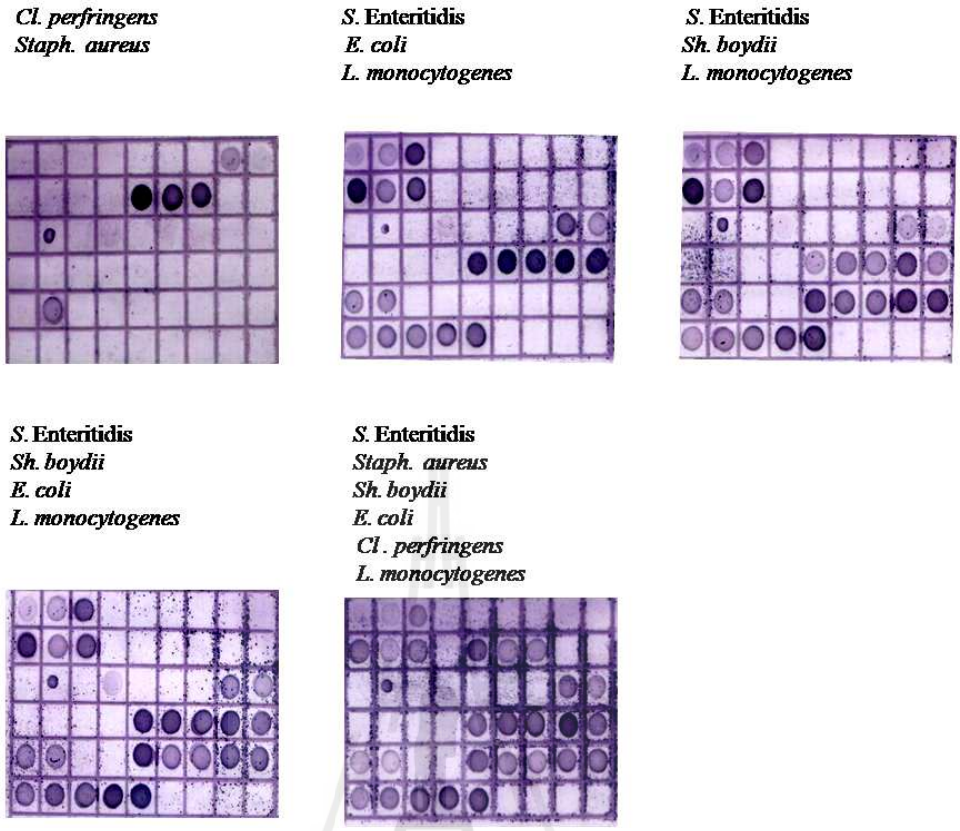


Figure 4.6 Multiple target bacteria detection by m-PCR-oligonucleotide array hybridization based method. Positions of specific probes on nylon membrane are shown in Figure 4.4A. Hybridization patterns of each target bacteria used in each test as label on top of each blot are combined in single array.

For the combination of conventional PCR and oligonucleotide array, probes targeted to specific genes (Table 4.3) were chosen for the detection sensitivity tests base on their efficiency and accuracy. Gene specific probes (3 probes for each target bacteria) were chosen for specific detection of each target bacteria including *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp.

4.3.3 Sensitivity of the oligonucleotide array detection

4.3.3.1 Sensitivity of the m-PCR-oligonucleotide array detection

The detection sensitivity of the assay was determined using genomic DNA mixture that was extracted from *S.* Enteritidis JCM 1652, *E. coli* TISTR 887, *Sh. boydii* DMST 28180 and *L. monocytogenes* DSM 12464. A 10-fold dilution series of genomic DNA mixtures ranging from 10-0.001 ng/µl were used as templates for m-PCR amplifications. Sensitivity of multiple target bacteria detection using m-PCR methods are shown in Figure 4.7A. Results from Figure 4.7A demonstrated that detection limit of these m-PCR for 4 target bacteria detection was 10 ng of each genomic DNA. Moreover, a 10-fold dilution series of genomic DNA mixture from 6 target bacteria including *S.* Enteritidis JCM 1652, *E. coli* TISTR 887, *Sh. boydii* DMST 28180, *L. monocytogenes* DSM 12464, *Cl. perfringens* isolate CP5, and *Staph. aureus* TISTR 517 were also tested to evaluate the efficiency of the developed m-PCR based method. Results of specific gene amplifications using 4 and 6 target bacteria as templates showed that just only 4 major amplicons could be separated (Figure 4.7). The efficiency of multiple target bacteria detection by m-PCR based methods seems decreased when number of target bacteria in the amplification reaction increased. Only faint band of *fim*Y, *ipa*H, and *prf*A were detected in agarose gel electrophoresis from amplifications of genomic DNA mixture at 10 ng of each genomic DNA extracted from 6 target bacteria (Figure 4.7B).

(A)

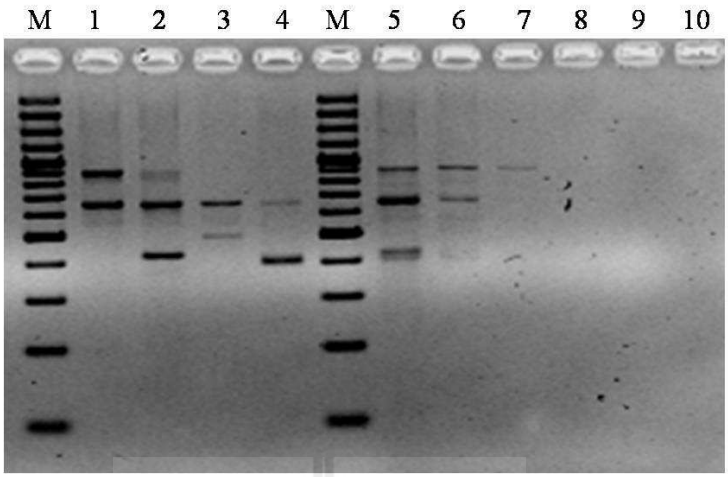


Figure 4.7 Sensitivity of m-PCR amplification. A series of 10-fold diluted genomic DNA mixture of 4 and 6 target bacteria were used as templates for m-PCR amplification. **(A)** A series of 10-fold diluted genomic DNA mixture of 4 target bacteria. Lanes: 1-4, 100 ng of genomic DNA template extracted from *E. coli* TISTR 887, *Sh. boydii* DMST 28180, *S.* Enteritidis JCM 1652, and *L. monocytogenes* DSM 12464, respectively; 5-9, A 10-fold series dilutions of the genomic DNA mixtures templates ranging from 10-0.001 ng of each genomic DNA, respectively; 10, negative control (H_2O). **(B)** A series of 10-fold diluted genomic DNA mixture of 6 target bacteria. Lanes: 1-5, A 10-fold series dilutions of the genomic DNA mixtures template ranging from 10-0.001 ng of each genomic DNA, respectively; 6, negative control (H_2O); 7-12, 100 ng of genomic DNA template extracted from *E. coli* TISTR 887, *Sh. boydii* DMST 28180, *S.* Enteritidis JCM 1652, *L. monocytogenes* DSM 12464, *Staph. aureus* TISTR 517, and *Cl. perfringens* isolate CP5, respectively.

(B)

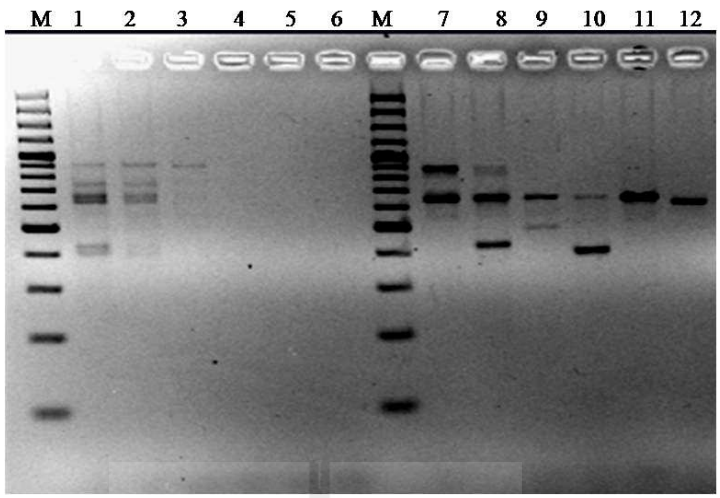


Figure 4.7 (Continued)

To distinguish all amplicons of m-PCR by oligonucleotide array, 10 µl of m-PCR products were labeled with 2 µl of DIG High Prime (Roche) and followed by hybridization with the specific probes on single array. The sensitivity for 4 target bacteria detection in pure culture by this assay was 1 ng of each genomic DNA (Figure 4.8A). For UA probes, the detection limit was 0.1 ng of each genomic DNA (Figure 4.8A). Compare to m-PCR detection by agarose gel, the m-PCR product amplified from mixture of 4 genomic DNA templates at concentration of 1 ng of each genomic DNA were not sufficiently separated and could not be observed all target genes amplicons on agarose gel (Figure 4.7A, Lanes 5-7). Sensitivity of m-PCR-oligonucleotide array could clearly differentiate all 6 target bacteria at as low as 10 ng of each genomic DNA (Figure 4.8B). Thus, m-PCR method followed by hybridization of the products to the oligonucleotide array would improve the detection sensitivity, accuracy and results interpretation of the m-PCR amplification for detection of more than 4 target bacteria in pure culture.

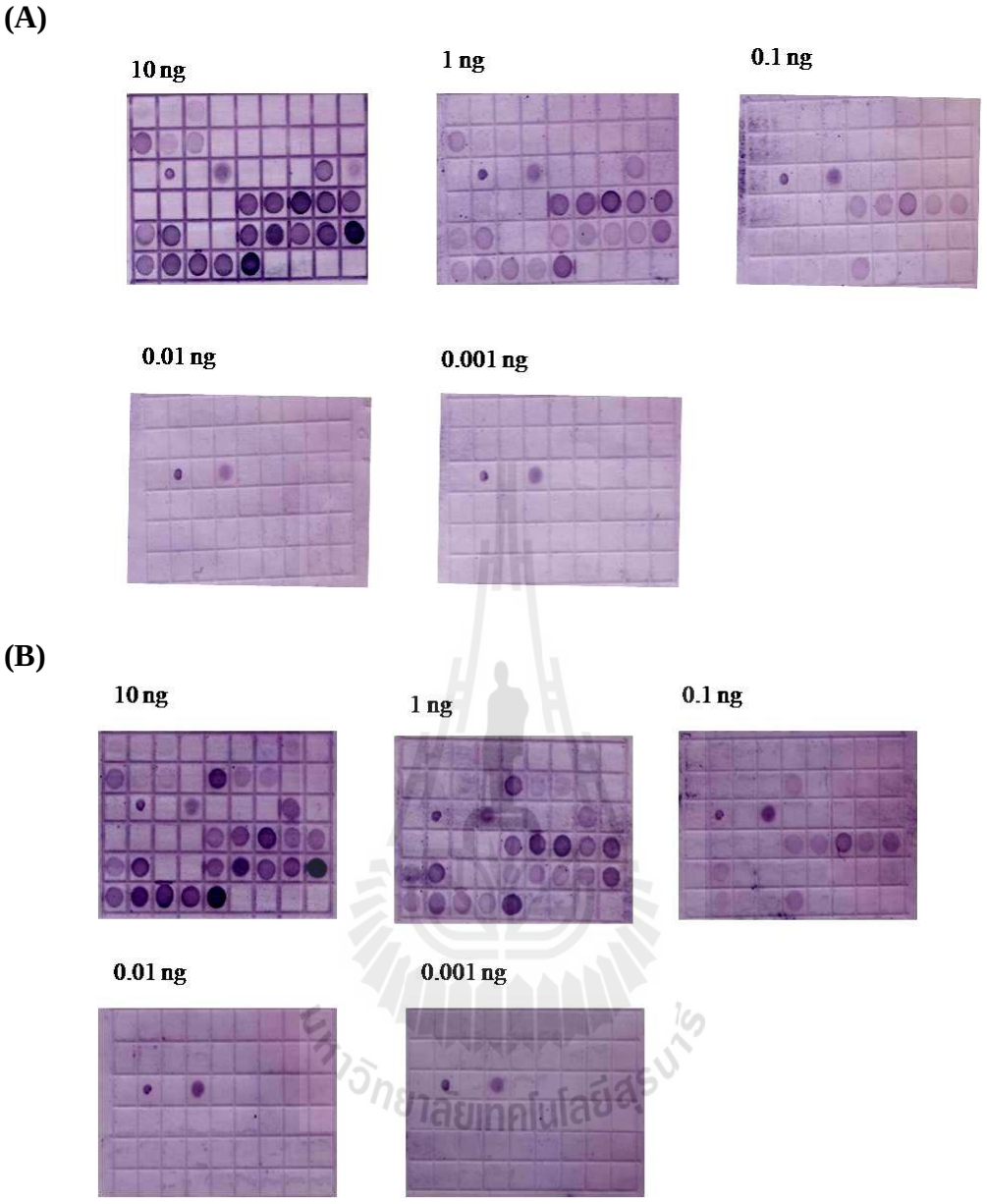
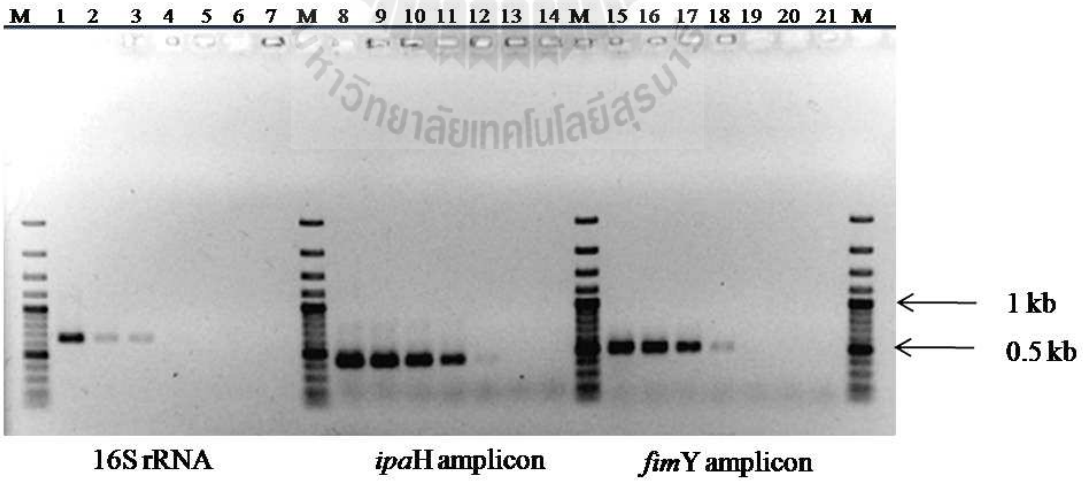


Figure 4.8 Sensitivity of m-PCR-oligonucleotide array for multiple target bacteria detection. Position of specific probes on nylon membrane is shown in Figure 4.4A. Genomic DNA extracted from each target bacteria were mixed at the same final concentration. A series of 10-fold diluted genomic DNA mixture of **(A)** 4 target bacteria and **(B)** 6 target bacteria ranging from 10-0.001 ng/µl were used as templates for m-PCR amplification followed by oligonucleotide array hybridization.

4.3.3.2 Sensitivity of the conventional PCR-oligonucleotide array detection

In this part, just 4 target bacteria including *S.* Enteritidis JCM 1652, *E. coli* TISTR 887, *Sh. boydii* DMST 28180 and *L. monocytogenes* DSM 12464 which were able to grow in the same condition were tested. To avoid less sensitivity of amplification by m-PCR, conventional PCR were also used to amplify each target gene. The sensitivity of multiple target bacteria detection by combination of conventional PCR and oligonucleotide array was evaluated using a mixture of genomic DNA (10^6 - 10^0 cells and 10-0.001 ng/µl of each) as templates. The PCR was able to amplify all of the 4 target genes with a detection limit of 10^6 cells for *prf*A gene, and 10^3 cells for *fim*Y, *ipa*H and *usp*A gene (Figure 4.9).

(A)



(B)

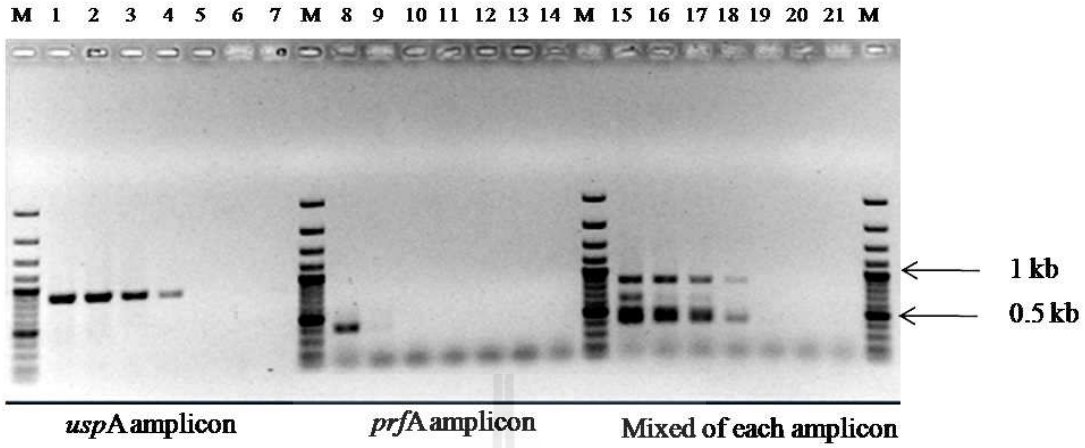


Figure 4.9 Sensitivity of conventional PCR amplification from different cells concentration (cells). **(A)** The amplification of 16S rRNA, *ipa*H, and *fim*Y gene with gene specific primers. Lanes: 1-7; 8-14; 15-21, five µl of 16S rRNA; *ipa*H; and *fim*Y gene amplified products from genomic DNA mixtures ranging from 10^6-10^0 cells of each target bacteria, respectively. **(B)** The amplification of *usp*A, and *prf*A gene with gene specific primers. Lanes: 1-7; 8-14, five µl of *usp*A; and *prf*A gene amplified products from genomic DNA mixtures ranging from 10^6-10^0 cells of each target bacteria, respectively. Lanes: 15-21, ten µl of mixed PCR product containing an equal volume of each amplified product amplified from genomic DNA mixture ranging from 10^6-10^0 cells of each target bacteria, respectively; M, DNA ladder 100 bp (Fermentas).

Compared to genomic DNA concentration in ng/µl of each target bacteria, the PCR was able to amplify *ipa*H and *usp*A gene with a detection limit of 0.01 ng of each genomic DNA, *fim*Y with detection limit of 0.1 ng of each genomic DNA and *prf*A with detection limit of 1 ng of each genomic DNA (Figure 4.10). However, agarose gel electrophoresis was unable to clearly separate all of the 5 amplicons (Figure 4.9B, lane 15-21, Figure 4.10B, lane 13-17) in mixed PCR products. Therefore, oligonucleotide array detection was performed by mixed an equal volume of each PCR product in a single tube and labeled them together. Ten µl of mixed PCR products were added with 2 µl DIG High Prime. All of the target bacteria were able to distinguish in a single array with specific probes as shown in Figure 4.11. The sensitivity of the oligonucleotide array was 10^5 cells (Figure 4.11B) which all 4 target bacteria could be detected. For *fim*Y, *ipa*H, and *usp*A probes, the detection limits were even lower (10^3 -10^2 cells of each target bacteria). Compared to genomic DNA concentration in ng/µl of each, the sensitivity of the oligonucleotide array was able to detect all of the 4 target bacteria with a detection limit of 0.1 ng. For 3 target bacteria detection including *E. coli*, *L. monocytogenes*, and *Sh. boydii*, detection limit was 0.01 ng of each genomic DNA (Figure 4.11C).

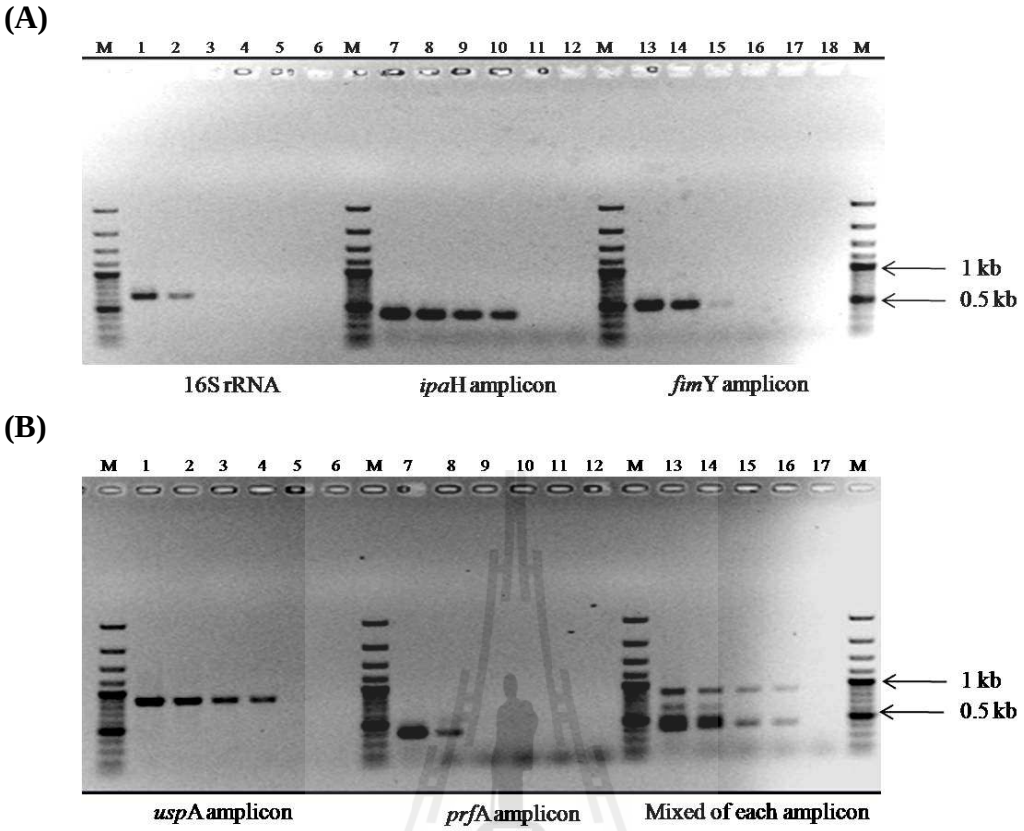


Figure 4.10 Sensitivity of conventional PCR amplification from different genomic DNA concentration (ng/µl). **(A)** The amplification of 16S rRNA, *ipa*H, and *fim*Y gene with gene specific primers. Lanes: 1-5; 7-11; 13-17, five µl of 16S rRNA; *ipa*H; and *fim*Y gene amplified products from genomic DNA mixtures ranging from 10-0.001 ng/µl of each genomic DNA extracted from each target bacteria, respectively. Lanes: 6; 12; 18, negative controls **(B)** The amplification of *usp*A, and *prf*A gene with gene specific primers. Lanes: 1-5; 7-11**,** five µl of *usp*A and *prf*A gene amplified products from genomic DNA mixtures ranging from 10-0.001 ng/µl of each genomic DNA extracted from each target bacteria, respectively. Lanes: 6; 12, negative controls. Lanes: 13-17, ten µl of mixed PCR products containing an equal volume of each amplified product amplified from genomic DNA mixture ranging from of 10-0.001 ng/µl of each genomic DNA extracted from each target bacteria, respectively. M, DNA ladder 100 bp (Fermentas).

(A)

SM 1	SM 2		CJ 1	CJ 2		SA 1	SA 2
FY 1	FY 2	FY 4		CP 1	CP 3	CP 4	
	P		16S			EC 1	EC 2
					UA 2	UA 3	UA 5
LM 1	LM 2			IH 1	IH 4	IH 5	
	PA 3	PA 4	PA 7				

Figure 4.11 Sensitivity of combination of conventional PCR-oligonucleotide array for multiple target bacteria detection**. (A)** Position of specific probes on nylon membrane. Positive controls are 0.1 ng of DIG-label control DNA (pBR328 DNA, linearized with *Bam*HI) (P) and 200 pmol 16S rDNA forward primer (16S). The abbreviated letters in grids are probes name as shown in Table 4.3.

(B)

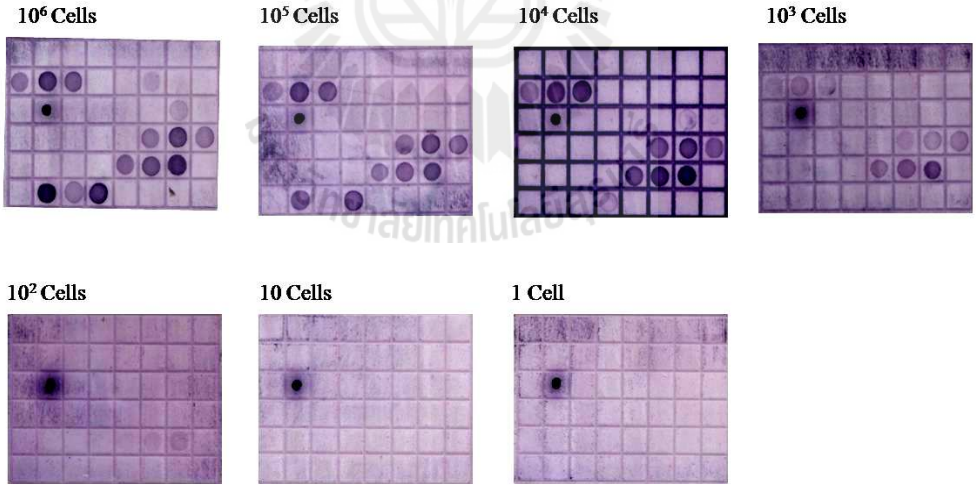


Figure 4.11 Sensitivity of combination of conventional PCR-oligonucleotide array for multiple target bacteria detection**.** Position of specific probes on nylon membrane is shown in Figure 4.11A. **(B)** Sensitivity of the oligonucleotide array for multiple foodborne pathogen detection in pure culture at different cells concentration (cells).

(C)

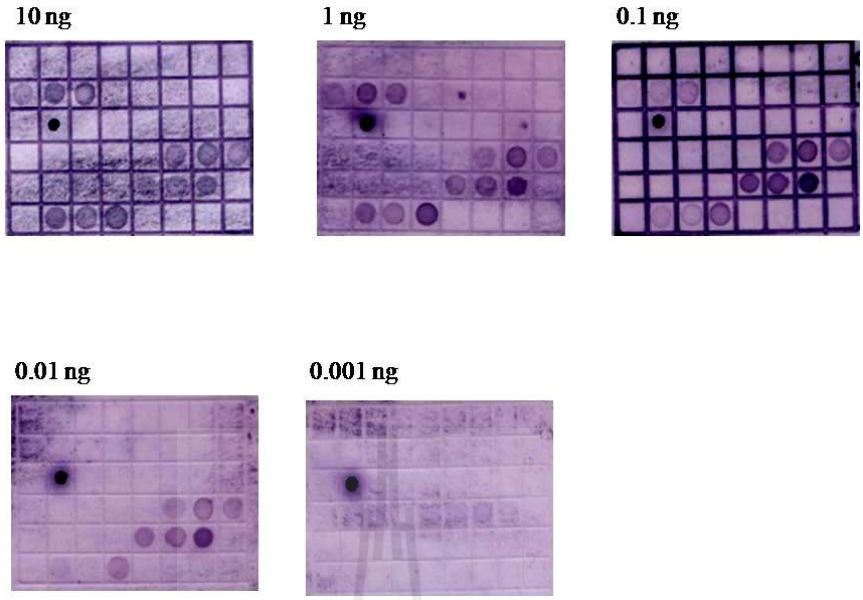


Figure 4.11 Sensitivity of combination of conventional PCR-oligonucleotide array for multiple target bacteria detection**.** Position of specific probes on nylon membrane is shown in Figure 4.11A. **(C)** Sensitivity of the oligonucleotide array for multiple foodborne pathogen detection in pure culture at different genomic DNA concentration (ng/µl).

4.3.4 Application of the oligonucleotide array for multiple foodborne pathogen detection in fresh chicken meat

The application of oligonucleotide array was tested with total of 10 fresh chicken meats including target bacteria spiked and non-spiked samples. In this experiment, 4 fresh chicken meat including 2 samples of chicken breast from open market, 1 sample of chicken wing and 1 sample of chicken thigh from supermarket in local area were tested as naturally and bacteria spiked sample. For spiked samples, 25 g of the each sample were spiked with 100 µl of each cell dilution solution including *S*. Enteritidis JCM 1652, *L. monocytogenes* DSM 12464, and *Sh. boydii* DMST 28180

at different final concentration ranging from 1-200 cells prior to adding of 225 ml pre-enrichment broth. Sample B1 and B2 were non-spiked chicken breast sample 1 and sample 2, respectively. Sample B1_1 and B2_1 were chicken breast sample B1 and sample B2 spiked with *L. monocytogenes* 1 cell, *Sh. boydii* 1 cell and *S.* Enteritidis 20 cells in 25 g sample, respectively. Samples B1_2 and B2_2 were chicken breast sample B1 and sample B2 spiked with *L. monocytogenes* 10 cells, *Sh. boydii* 3 cells and *S.* Enteritidis 200 cells in 25 g sample, respectively. Sample T1 was non-spiked chicken thigh sample 1; T1_1 was chicken thigh sample T1 spiked with *L. monocytogenes* 20 cells, *Sh. boydii* 80 cells and *S.* Enteritidis 8 cells in 25 g sample. Sample W1 was non-spiked chicken wing sample 1. Sample W1_1 was chicken wing sample W1 spiked with *L. monocytogenes* 20 cells, *Sh. boydii* 80 cells and *S.* Enteritidis 8 cells in 25 g sample. The enrichment steps were performed to increase target bacterial cells in all samples as described in materials and methods (4.2.6). Total genomic DNA were extracted from each enrichment culture of each samples and used for multiple target bacteria detection using combination of m-PCR-oligonucleotide array and conventional PCR-oligonucleotide array.

4.3.4.1 M-PCR-oligonucleotide array detection

In real samples application, m-PCR conditions were optimized again for amplification of all target genes from real sample in a single reaction. The optimum amplification of multiple target bacteria using m-PCR were 0.032 µM *ipa*H and *usp*A, 0.036 µM *fim*Y, 0.28 µM *prf*A and 0.14 µM 16S rRNA (internal control). With these conditions, at least 4 amplicons were found from the amplification of large number of target bacteria in mixed genomic DNA from enrichment culture. The amplification of total genomic DNA mixture extracted from enrichment culture of

each chicken meat samples are shown in Figure 4.12, 4.13 and 4.14. Mostly m-PCR products observed from food samples application showed that the separations of all mixed amplicons were difficult and not sufficient on 4% agarose gel.

All samples showed amplicons of *fim*Y gene both in reaction using genomic DNA extracted from only RV, TT culture as templates and mixed genomic DNA templates of several enrichment cultures. However, the detection of *prf*A and *ipa*H amplicons were very poor and ambiguous because the size of these PCR products were only 20 bp different. No bands of *prf*A gene amplification was observed from the m-PCR amplification using genomic DNA extracted from Fraser culture as template in samples inoculated with *L. monocytogenes* at very low initial contamination level of 1 or 10 cells in 25 g sample (Figure 4.12A lane 5 and Figure 4.12B lane 4) (Sample B1_1 and B1_2). Moreover, the efficiency of combining the m-PCR with oligonucleotide array for detection of *L. monocytogenes* at low contamination level in sample decreased when all genomic DNA extracted from each enrichment culture were mixed together and used as templates. With this reasons, only genomic DNA extracted from Fraser culture was used as templates for m-PCR amplification alone followed by oligonucleotide array hybridization assay to confirm the results of *L. monocytogenes* detection.

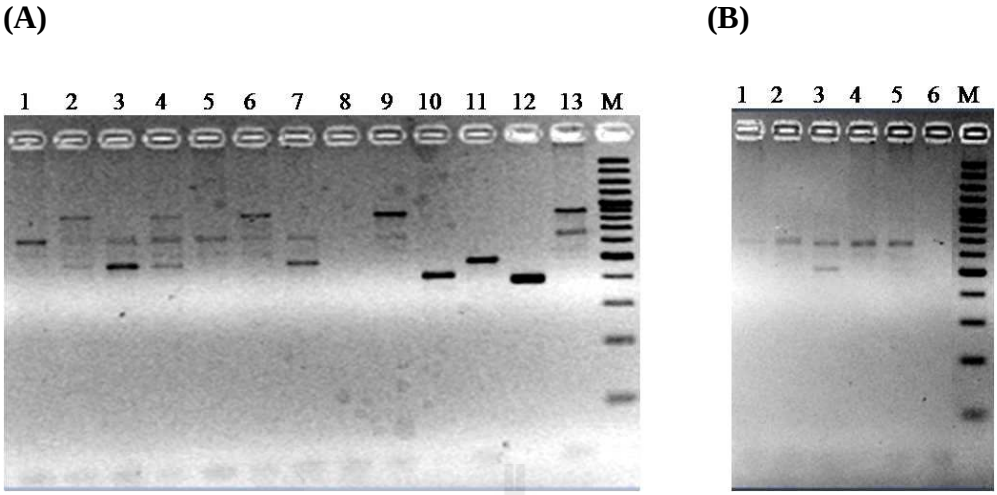


Figure 4.12 M-PCR amplified products of total genomic DNA extracted from enrichment cultures including Fraser broth (F), RV broth (RV), TT broth (TT), Mixed of Shigella broth (Sh), BPW, RV, TT and F (Mixed) of sample B1, B1_1, and B1_2. **(A)** Lanes: 1-4, m-PCR products of sample B1 amplified from F, RV, TT, and Mixed, respectively; Lanes: 5-8, m-PCR products of sample B1_2 amplified from F, RV, TT, and negative control (H_2O), respectively; Lanes: 9-13, m-PCR product amplified from *E. coli*, *Sh. boydii*, *S.* Enteritidis, *L. monocytogenes*, and m-PCR products of sample B 1_2 amplified from mixed templates, respectively. **(B)** Lanes: 1-6, m-PCR products of sample B1_1 amplified from Sh, RV, TT, F, Mixed, and negative control (H_2O), respectively; M, 100 bp DNA marker (Fermentas).

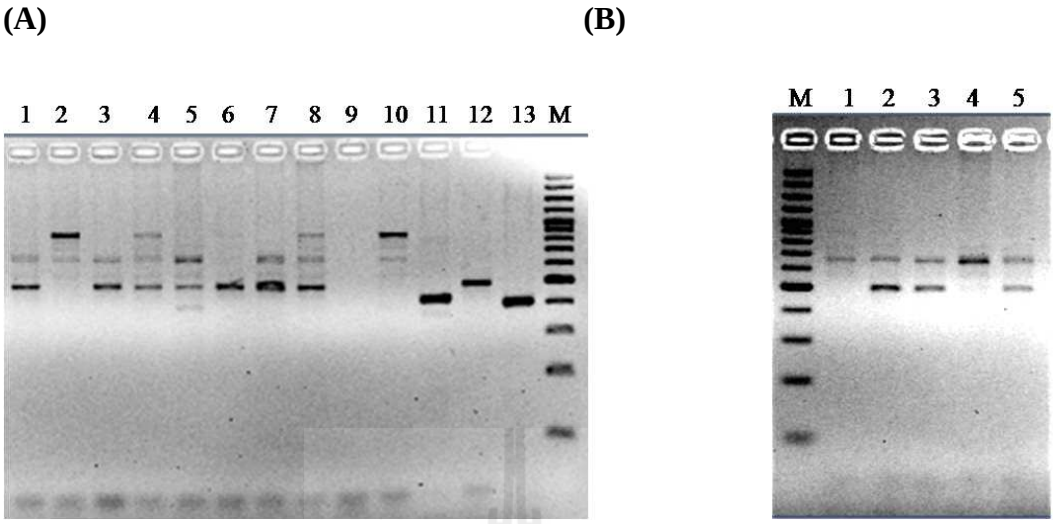


Figure 4.13 M-PCR amplified products of total genomic DNA extracted from enrichment cultures including Fraser broth (F), RV broth (RV), TT broth (TT), Mixed of Shigella broth (Sh), BPW, RV, TT and F (Mixed) of sample B2, B2_1, and B2_2. **(A)** Lanes: 1-4, m-PCR products of sample B2 amplified from F, RV, TT, and Mixed, respectively; Lanes: 5-9, m-PCR products of sample B2_2 amplified from F, RV, TT, Mixed, and negative control (H_2O), respectively; Lanes: 10-13, m-PCR product amplified from *E. coli*, *Sh. boydii*, *S.* Enteritidis, *L. monocytogenes*, respectively. **(B)** Lanes: 1-5, m-PCR products of sample B2_1 amplified from Sh, RV, TT, F, and Mixed, respectively; M, 100 bp DNA marker (Fermentas).

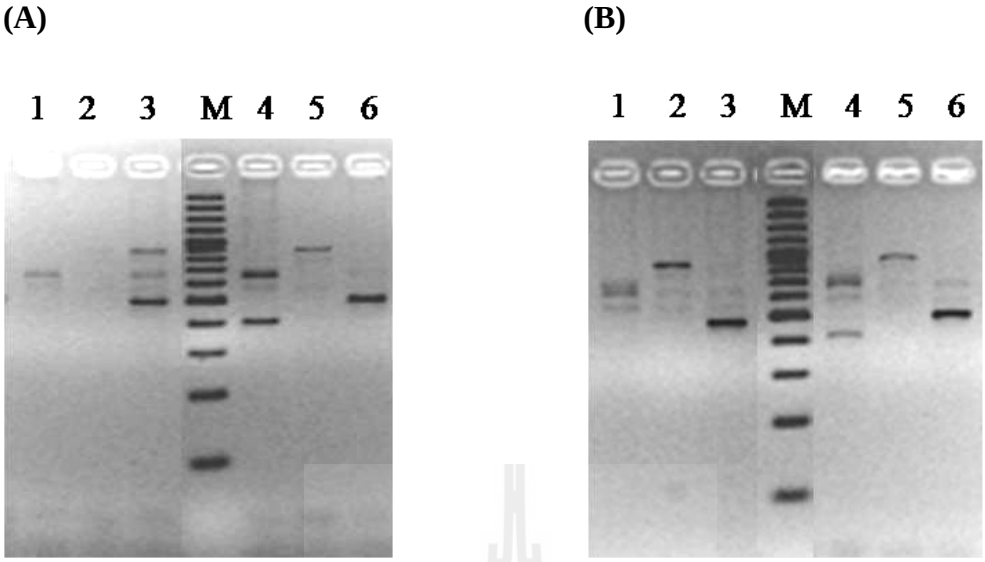


Figure 4.14 M-PCR amplified products of total genomic DNA extracted from enrichment cultures including Fraser broth (F), RV broth (RV), TT broth (TT), Mixed of Shigella broth (Sh), RV, and TT (Mixed) of sample W1, W1_1, T1, and T1_1. **(A)** Lanes: 1-3, m-PCR products of sample W1 amplified from F, Sh, and Mixed, respectively; Lanes: 4-6, m-PCR products of sample W1_1 amplified from F, Sh, and Mixed, respectively. **(B)** Lanes: 1-3, m-PCR products of sample T1 amplified from F, Sh, and Mixed, respectively; Lanes: 4-6, m-PCR products of sample T1_1 amplified from F, Sh, and Mixed, respectively; M, 100 bp DNA marker (Fermentas).

Results of multiple target bacteria detection from each sample by combination of m-PCR and oligonucleotide array are shown in Figure 4.15 and 4.16. For *L. monocytogenes* detection, the contamination of *L. monocytogenes* was found in only 1 of the non-spiked samples (sample B2) (Table 4.5). These results agree with

the results of presumptive colonies-m-PCR tests. Twenty presumptive colonies were collected from selective agar and tested. Results showed that only 1 of them were positive for *prf*A amplicons. These results indicated that sample B2 was contaminated with *L. monocytogenes* and another *Listeria* species. After identification of all *Listeria* isolates using carbohydrate utilization and motility testes as described in Chapter 2, results indicated that this sample was contaminated with *L. monocytogenes* (*prf*A positive) and *L. innocua*.

In sample B1, weak hybridization signal of PA probes were detected when 10 cells of *L. monocytogenes* were inoculated (Figure 4.15A, sample B1_2 F). However, strong hybridization signal of PA probes could be detected in sample B2, B2_1, B2_2, W1_1, and T1_1 because they were contaminated with initial concentration of *L. monocytogenes* at more than 10 cells in 25 g of those samples. These results indicated that the sensitivity of the detection for *L. monocytogenes* using combination of m-PCR-oligonucleotide array in food samples were at least 10 cells in 25 g samples. No cross-reactivity of PA probes with m-PCR products amplified from other non-target bacteria in enrichment culture was confirmed because negative hybridization signal of these probes in samples W1 and T1 with no *L. monocytogenes* inoculation was observed. In samples B2_1, W1, T1 and T1_1, results of carbohydrate utilization and motility profiles analysis indicated that they were *L. monocytogenes* or *L. innocua*. After combining the results with the amplification of each colony with m-PCR, no *prf*A amplicons were detected from all tested isolates. These results indicated that only *L. innocua* were detected in those samples.

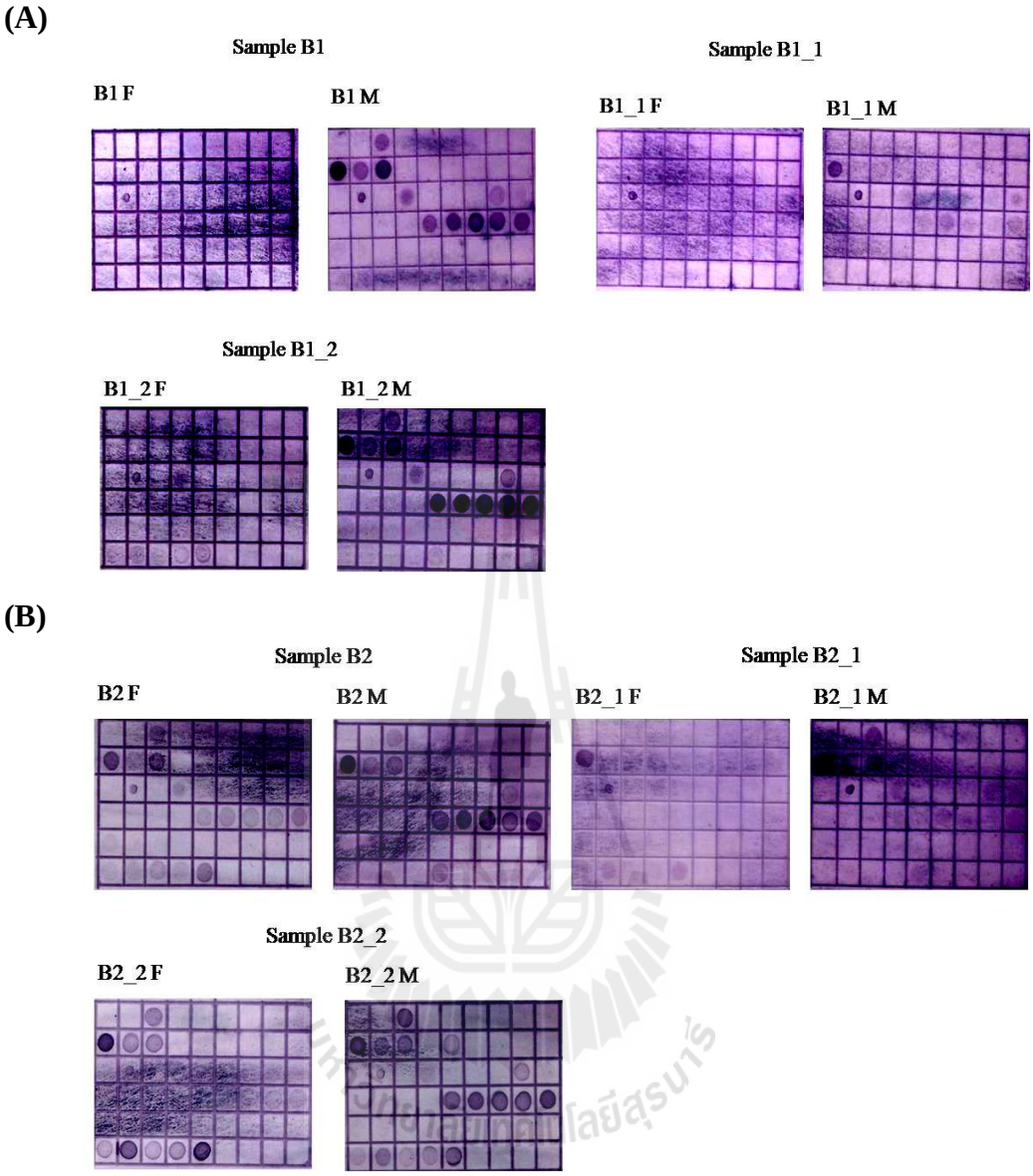
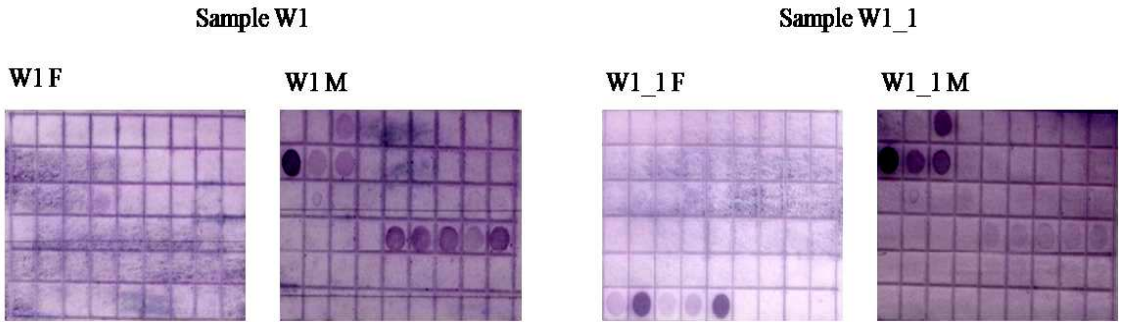


Figure 4.15 m-PCR-oligonucleotide array hybridization results of **(A)** sample B1, B1_1, and B1_2 and **(B)** sample B2, B2_1, and B2_2. Ten µl of labeled m-PCR products amplified from total genomic DNA extracted from Fraser culture (F) and Mixed (M) (genomic DNA mixture obtained from Shigella culture (Sh), RV, TT, BPW and F) were separately hybridized with specific probes on array. Probes positions are shown in Figure 4.4A. Hybridization patterns of m-PCR products from Fraser culture (F) and mixed enrichment culture (M) were combined for 4 target bacteria detection for each sample.

(A)



(B)

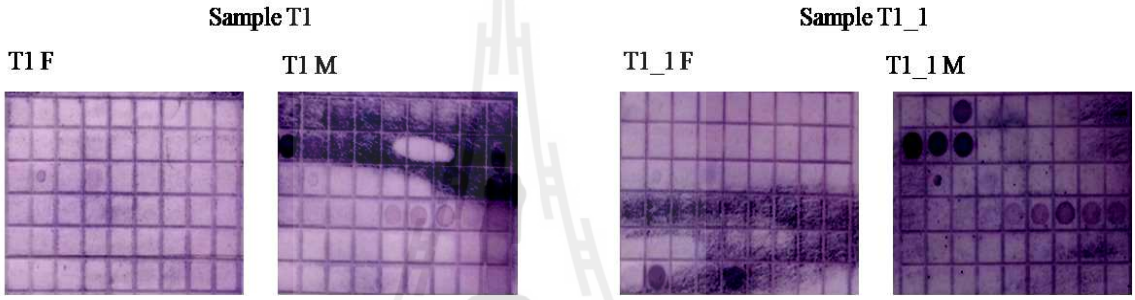


Figure 4.16 m-PCR-oligonucleotide array hybridization results of **(A)** sample W1, W1_1, **(B)** T1, and T1_1. Ten µl of labeled m-PCR amplified from total genomic DNA extracted from Fraser culture (F) and mixed (M) (genomic DNA mixture obtained from Shigella culture (Sh), RV, TT) were separately hybridized with specific probes on array. Probes positions are shown in Figure 4.4A. Hybridization patterns of m-PCR products from Fraser culture (F) and mixed enrichment culture (M) were combined for 4 target bacteria detection for each sample.

Results of multiple target bacteria detection using combination of m-PCR-oligonucleotide array hybridization and conventional culture are summarized in Table 4.5. The prevalence of *Salmonella* and *E. coli* were found in all samples including spiked and non-spiked samples which could be detected by oligonucleotide array and confirmed by colonies m-PCR. From total of 10 samples, 3 target bacteria could be simultaneously detected from 6 samples, 2 target bacteria from 4 samples (Table 4.5). When comparing the conventional culture method to the array, 3 target bacteria could be detected from only 2 of 6 spiked samples while oligonucleotide array could detect 3 target bacteria simultaneously from 5 of 6 spiked samples (Table 4.5).

All results in these experiments indicated that using array hybridization patterns of DNA obtained from Fraser culture and mixture of all enrichment cultures could simultaneously detected *L. monocytogenes*, *E. coli*, and *Salmonella* sp. in fresh chicken meat samples. However, weak hybridization signal of some probes such as UA probes were found in some samples (B1_1 M, B2_1 M, W1_1 M). These problems might be due to the less efficiency of multiple target genes amplification using m-PCR in sample containing genomic DNA of non-target bacteria. To prove these hypothesis, amplification of the same templates were performed with single primer pair by conventional PCR in next part.

Table 4.5 Application of m-PCR-oligonucleotide array for foodborne pathogen detection from fresh chicken meat samples

Samples	m-PCR-oligonucleotide array test	Colony confirmation from the isolation agar [a]
B1	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
B1_1	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
B1_2	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp.
B 2	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp. *L. monocytogenes*
B2_1	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp.
B2_2	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp. *L. monocytogenes*
W1	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
W1_1	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp. *L. monocytogenes*
T1	*E. coli* *Salmonella* sp.	*E. coli* *Salmonella* sp.
T1_1	*E. coli* *Salmonella* sp. *L. monocytogenes*	*E. coli* *Salmonella* sp.

[a] **Colony confirmation;** presumptive colonies on selective agar from each sample was confirmed by m-PCR. For *L. monocytogenes* confirmation, more than 10 presumptive colonies were collected from selective agar and tested for carbohydrate utilization and motility test. Results of colony-m-PCR (positive for *prf*A amplicon) and characteristic of carbohydrate utilization and motility tests were combined for *L. monocytogenes* identification.

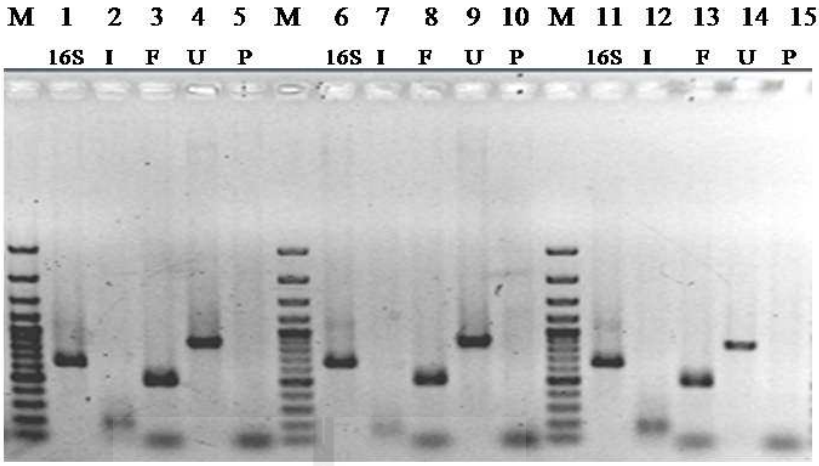
4.3.4.2 Conventional PCR-oligonucleotide array detection

The application of conventional PCR-oligonucleotide array was tested using genomic DNA extracted from enrichment culture of 10 fresh chicken meat including target bacteria spiked and non-spiked samples as used in m-PCR-oligonucleotide array part. To avoid false negative results from the enrichment conditions, an enrichment step with suitable and specific enrichment medium were used for each pathogen. Both spiked and non-spiked samples were examined for all target bacteria using selective agar and the presumptive colonies were confirmed by colony-m-PCR. For amplification of target genes, an equal volume of genomic DNA extracted from enrichment culture including BPW, RV, TT, Fraser, and Shigella broth were mixed. One µl of mixed DNA was used as template for amplification of 16S rRNA, *usp*A, *fim*Y, and *ipa*H genes separately. For the amplification of *prf*A gene, genomic DNA template extracted from Fraser culture was used as template alone. Results of the PCR amplifications from each sample are shown in Figure 4.17 and 4.18. Amplification of each target genes from genomic DNA mixture of several enrichment culture showed that the expected size of single band were found in all samples when single pair of specific primers were used. Specific detection of *Salmonella* and *E. coli* using *fim*Y and *usp*A genes as target showed amplification of *fim*Y and *usp*A genes detected from all samples. For the detection of *Shigella*, *ipa*H amplicon could not be detected on agarose gel in the sample of non-spiked or spiked with very low number of cell concentration (1-3 cells in 25 g samples) (Figure 4.17A and B). These results indicated that m-PCR and conventional PCR were unable to detect *Shigella* in sample contaminated with very low initial cell concentration. Sample spiked with *L. monocytogenes* and *Sh. boydii* at high level of initial cell

concentration such as sample W1_1 and T1_1 showed that expected amplicons of *prf*A and *ipa*H were detected while non-spiked samples (W1 and T1) could not be observed these amplicons (Figure 4.18A and B lane 2 and 5). Compared this results with m-PCR amplification, the *ipa*H amplicon could not be detected in the same sample using the same mixed genomic DNA template for m-PCR amplification. These results indicated that the m-PCR was less sensitive for simultaneously amplification of several target genes and could give false negative results.

However, only the present of the gene specific amplicon was not enough to confirm the results interpretation of the target bacteria detection since the size separation of each amplicon is not clear. Therefore, to improve the sensitivity and accuracy of the multiple pathogen detection of real food samples with high background of non-target bacteria, PCR validation steps are still required. Oligonucleotide array were used to identify each amplicon in a single reaction. For the detection of *L. monocytogenes* in this investigation, directly amplification of *prf*A gene from genomic DNA mixture was less sensitive than the other. Therefore, 1 µl of genomic DNA extracted from Fraser culture was used as template for *prf*A gene amplification. Results of multiple target bacteria detection using single array hybridization of each sample are shown in Figure 4.19. Strong hybridization signal were found for specific detection of all 4 target bacteria in a single array of samples spiked with target bacteria at high initial concentration of cell (Figure 4.19, sample W1_1 and T1_1). Specific hybridization patterns observed from each array could be used to identify more than 3 target bacteria in samples with unambiguous and high accuracy.

(A)



(B)

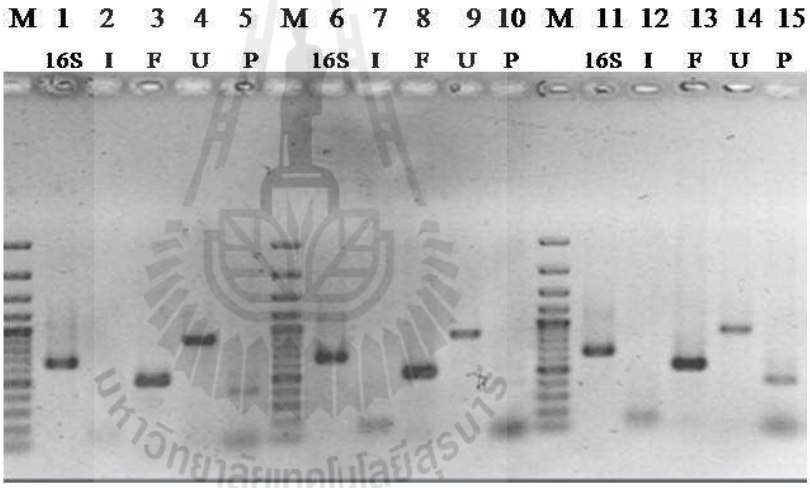
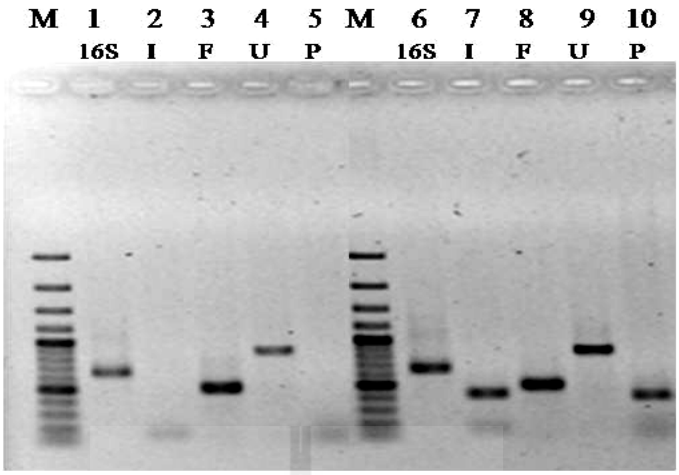


Figure 4.17 Convention PCR amplification of each target gene including 16S rRNA (16S), *ipa*H (I), *fim*Y (F), *usp*A (U), and *prf*A (P) as labeled on each lane using mixed genomic DNA of each enrichment culture (Sh, RV, TT, BPW, and Fraser culture) and only Fraser culture (for amplification of *prf*A gene) as templates. **(A)** Lanes: 1-5; 6-10; 11-15, PCR products of sample B1; B1_1; and B1_2, respectively. **(B)** Lanes: 1-5; 6-10; 11-15, PCR products of sample B2; B2_1; and B2_2, respectively; M, 100 bp DNA marker (Fermentas).

(A)



(B)

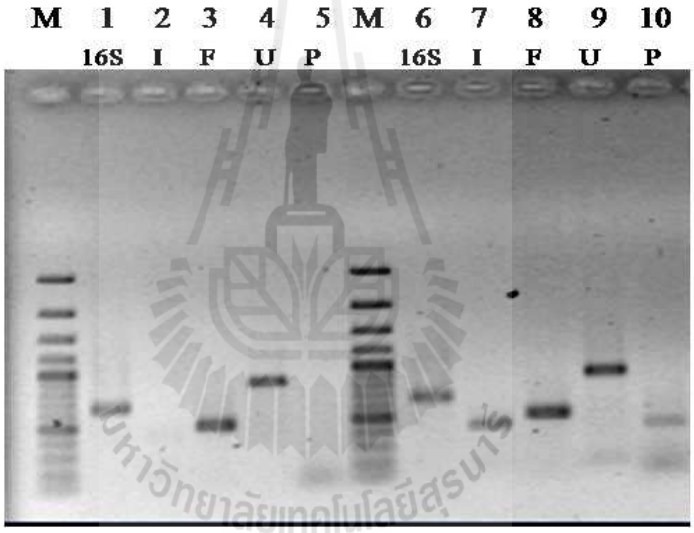


Figure 4.18 Convention PCR amplification of each target gene including 16S rRNA (16S), *ipa*H (I), *fim*Y (F), *usp*A (U), and *prf*A (P) as labeled on top of each lane using mixed genomic DNA of each enrichment culture (Sh, RV, TT, and Fraser culture) and only Fraser culture (for amplification of *prf*A gene) as templates. **(A)** Lanes: 1-5; 6-10, PCR products of sample W1; and W1_1, respectively. **(B)** Lane: 1-5; 6-10, PCR products of sample T1; and T1_1, respectively; M, 100 bp DNA marker (Fermentas).

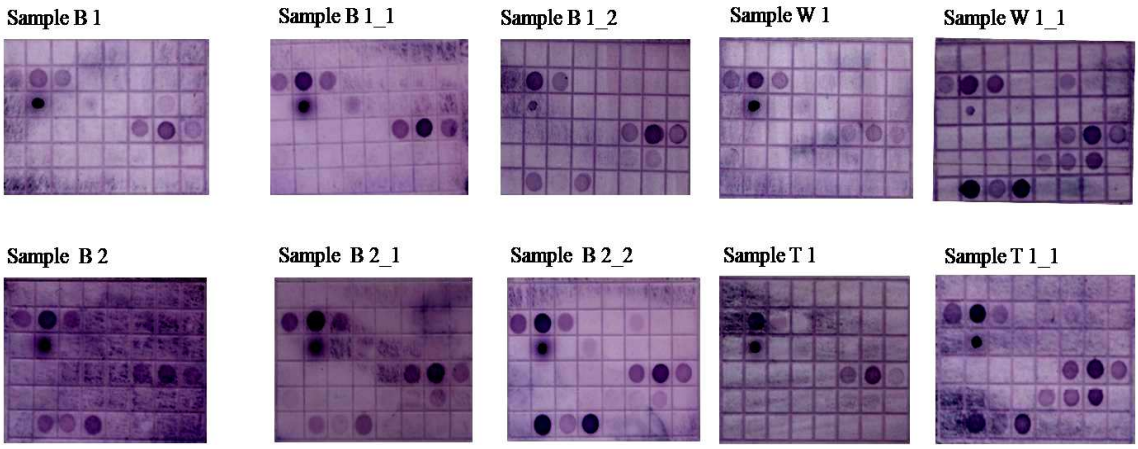


Figure 4.19 Conventional PCR-oligonucleotide array hybridization results of multiple foodborne pathogen detection in fresh chicken meat samples. PCR products of each target gene amplification obtained from each sample were mixed and labeled. The labeled PCR products were hybridized with specific probes on single array. Probe positions of array are shown in Figure 4.11A.

The positive signals observed from each probes of each samples were scored to be positive (+) and compared results with conventional culture detection as summarized in Table 4.6. The prevalence of *Salmonella* and *E. coli* were found in all 4 non-spiked samples. One non-spiked sample (B2) was contaminated with *L. monocytogenes*. In this research, no presumptive colonies of *Shigella* were observed from all non-spiked and spiked samples. Only one (B2_1) from 2 spiked samples (B1_1, B2_1) with 1 cell of *Sh. boydii* in 25 g can detected positive *ipa*H signal by oligonucleotide array hybridization (Figure 4. 19). This research could not detected *L. monocytogenes* in 25 g sample (B1_1) using neither oligonucleotide array nor conventional culture when initial final concentration was 1 cell.

Considering the non-spiked sample without *L. monocytogenes* and *Shigella* contamination, sensitivity of *L. monocytogenes* and *Sh. boydii* detection using conventional PCR-oligonucleotide array were at least 10 cells (sample B1_2, W1_1, T1_1) and 3 CFU (B1_2, B2_2, W1_1, T1_1) of initial contamination in 25 g samples, respectively. At very low number of cell inoculation of *L. monocytogenes* and *Sh. boydii* in some samples, positive signal could be observed from probes of *prf*A (sample B1_2), and *ipa*H (B1_2, B2_1, and B2_2) but the PCR product in agarose gel could not be observed. This result indicated that the oligonucleotide array is able to increase the detectability compared to the PCR method alone. However, 16S rRNA gene show weak or negative signal for most hybridization analysis. In conclusion, from 10 samples, 2, 3, and 4 target bacterial pathogens could be simultaneously detected from 4, 1 and 5 samples, respectively (Figure 4.19 and Table 4.6). These results made clear that the conventional PCR-oligonucleotide array could be used to detect all 4 target bacteria with more rapid, more accuracy, easier, and more labor saving than that using the conventional culture method.

Table 4.6 Application of conventional PCR-oligonucleotide array for multiple foodborne pathogens detection in fresh chicken meat samples

Sample	Targeted bacteria/ Target genes/ Probes/ [a]																		Colony confirmation from the selective agar [b]
	E. coli					*Shigella* spp.			*Salmonella* spp.					*Listeria* spp.		*L. monocytogenes*			
	16S rRNA		*usp*A			*ipa*H			16S rRNA		*fim*Y			16S rRNA		*prf*A			
	EC1	EC2	UA2	UA3	UA5	IH1	IH4	IH5	SM1	SM2	FY1	FY2	FY4	LM1	LM2	PA3	PA4	PA7	
B 1	+	-	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	*E. coli* *Salmonella* sp.
B 1_1	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	*E. coli* *Salmonella* sp.
B 1_2	-	-	+	+	+	-	+	+	-	-	+	+	+	-	-	+	+	+	*E. coli* *Salmonella* sp.
B 2	-	-	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	+	*E. coli* *Salmonella* sp. *L. monocytogenes*
B 2_1	-	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	*E. coli* *Salmonella* sp.
B 2_2	-	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	*E. coli* *Salmonella* sp. *L. monocytogenes*
W1	-	-	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	*E. coli* *Salmonella* sp.
W 1_1	-	-	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	*E. coli* *Salmonella* sp. *L. monocytogenes*
T 1	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	*E. coli* *Salmonella* sp.
T 1_1	-	-	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	*E. coli* *Salmonella* sp.

[a] **Symbols**: + : positive hybridization signal; - : negative hybridization signal

[b] **Colony confirmation**: presumptive colony on the selective agar from each sample was confirmed by m-PCR. For *L. monocytogenes* confirmation, more than 10 presumptive colonies were collected from selective agar and tested for carbohydrate utilization and motility test. Results of colony-m-PCR (positive for *prf*A amplicon) and characteristic of carbohydrate utilization and motility tests were combined for *L. monocytogenes* identification.

4.4 Discussion

The conventional methods for detecting enteropathogens involved isolation follow by biochemical identification for each pathogen. They are very laborious and time consuming (De Boer and Beumer, 1999; You et al., 2008). Furthermore, processing of large numbers of samples is not easy in general, 10 or more tests may be necessary for differentiation of the species within a group (Settanni and Corsetti, 2007). Therefore, rapid, specific, and sensitive methods for detecting and identifying pathogens have been developed. In this study, combination of oligonucleotide array and m-PCR or conventional PCR targeted to specific genes were developed and successfully used for specific detection of dominant foodborne pathogens and food safety indicator in chicken meat including *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., and *E. coli*. Advantages of both oligonucleotide array and m-PCR for multiple pathogens detection are that multiple target bacteria can be detected simultaneous. By these methods, labor, cost, and identification time can be reduced. Although variations of the physiological characteristic were found from the isolated strains used in this study, both methods can be used to specifically identify the target bacteria (Figure 4.4).

Digoxigenin-linked enzyme color development method was used in this study for oligonucleotide array assay. This system does not need any special or expensive equipment for material array construction nor signal detection. By this reasons, multiple pathogen detection using this system is cheap, easy and suitable to apply in general molecular laboratories compared to fluorescence or other colorimetric development methods. In previous reports using (DIG) or biotin for oligonucleotide array assay, only conserve gene including 16S rRNA gene (Chiang et al., 2006), 23S

rRNA gene (Hong et al., 2004) and *gro*EL gene (Hu et al., 2011) were selected as targets. Detections of multiple pathogens were done in pure culture, food sample, and clinical samples (Hong et al., 2004; Chiang et al., 2006; Hu et al., 2011). However, problems of low discriminatory among target and non-target bacteria were reported. In this study, the accuracy of probes targeted to 16S rRNA gene was low for *E. coli* and *Salmonella* spp. detection (Chapter 3, Table 3.4). Therefore, in this part, primers and probes for identifying 4 target bacteria were also designed against genes specifically found in the respective pathogens to prevent false-positive or false-negative results.

The specificity of oligonucleotide array detection mainly depends on the selection of gene targets and the design of oligonucleotide array probes (Suo et al., 2010). To save cost and time for probe analysis, m-PCR conditions were developed and optimized for all target genes amplification in single reaction. Target genes used for probes design in this works were 16S rRNA genes and species or genus specific genes. Specificity and accuracy of oligonucleotide array probes for detection of multiple foodborne pathogens were tested with several reference and isolated strains. In the m-PCR reaction, the absence of a PCR product when an enrichment medium is used as the source of template DNA could be due to either the absence of target DNA or an amplification failure due to the presence of inhibitors (Villalobo and Torres, 1998). In our research, the 16S rRNA gene which can be amplified from all target bacteria was used as an internal control of the presence of amplifiable bacterial DNA. Therefore, amplification of 16S rRNA in this research was not only used as internal control for m-PCR amplification reaction but also used for identification some target bacteria in genus level as shown in Chapter 3. As all available data now, this is the

first report to combine these target genes (16S rRNA, *usp*A, *prf*A, *fim*Y, and *ipa*H) together for specific detection of *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *Shigella* spp. in a single reaction of m-PCR.

Gene specific primers from previously published data were also evaluated for specificity. The cross-amplification of the *usp*A gene, encodes for a highly conserved universal stress protein present in all *E. coli* (Chen and Griffiths, 1998), were found in this investigation. In 2007, Chen and colleagues has shown that within the 800 bp of *usp*A sequences, only 17 bp mismatches were found between the *usp*A of *Sh. sonnei* and that of *E. coli* K-12 (Chen, 2007). My work here showed that this gene can be amplified not only from *E. coli* but also from all 4 *Shigella* species found in Thailand due to the high identity of the gene between *E. coli* and *Shigella* sp. (Chen, 2007). However, the *usp*A gene was shown to be conserved among all *E. coli* isolates and can be used for differentiation of *E. coli* and non-*E. coli* bacteria from the enrichment culture such as isolate RV3 (Chapter 2, Table 2.4). The *usp*A and *ipa*H gene amplification of isolate RV3 (Figure 4.2 lane 6) which has the 16S rDNA gene hybridization pattern similar to *E. coli* was negative. This result indicated that isolate RV3 should not be *E. coli* or *Shigella* sp. Therefore, this *usp*A gene was still used as the target for *E. coli* detection using m-PCR amplification.

The previously published primers for specific detection of *Shigella* were evaluated in this investigation. Primers for amplification of *vir*A gene (Mao et al., 2008) gave negative results to *Shigella* sp. isolated strains (Sh1). The *vir*A gene located on virulence plasmids of Shigellae (Gall et al., 2005), thus this gene might be lose in some isolated strains. In contrast, detection of *Shigella* spp. using *ipa*H as target showed that this gene was conserve among all *Shigella* isolates include isolate

Sh1. The *ipa*H gene is encoded on a 220 kb plasmid and also present on the chromosome (Ashida et al., 2007). By this reasons, *ipa*H gene was more conserve for all *Shigella* isolates compared to other genes. Negative results observed from *vir*A gene amplification in isolate Sh1 indicated that some published primer targeted to specific genes might not be applied for detection of some local isolated strains. Thus false negative for foodborne pathogen detection can occur.

The amplification efficiency of the gene specific primers in mixed primer set in m-PCR reaction is also an important point that influences the accuracy of the technique. The amplification ability of *inv*A (Mao et al., 2008) and *fim*Y primers in m-PCR reactions for specific detection of *Salmonella* spp. was investigated. Compared to *fim*Y primers, lower yields of PCR products were obtained when *inv*A primers were used. This might be due to the compatibility of *inv*A primers with the mixed primer set in the m-PCR reaction was lower than that of *fim*Y primers. Therefore, the suitable target genes for specific detection of 4 target bacteria in this part were *fim*Y, *ipa*H, *prf*A, and *usp*A genes.

The detection capability of m-PCR was still limited due to the less sensitive and low resolution of agarose gels for traditional PCR detection (Figure 4.6 and 4.7). Sensitivity of the PCR based methods was lower than that of oligonucleotide array hybridization (Hong et al., 2004; Chiang et al., 2006). Moreover, results interpretation using only the sizes of target PCR product on agarose gel might be ambiguous especially if several targets were amplified in a single reaction. Therefore, combining these methods with oligonucleotide array could increase detectability and accuracy of the detection systems which can be applied to detect multiple target bacterial species directly from enrichment culture. One of the major advantages of the oligonucleotide

array assay over agarose gel analysis of the PCR products was that detection did not rely solely on the length of the PCR products, but also required the fragments to contain sequences that were complementary to the oligonucleotide probes on the microarray (Kim et al., 2010).

Sensitivity of detection is important criteria to evaluate the suitable methods for pathogens detection. Sensitivity of all 4 target bacteria detection by m-PCR-oligonucleotide array in this works was 1 ng of each genomic DNA extracted from pure cultures which corresponds to approximately 2×10^5 copies of the bacterial genome and was equivalent to 10^4 cells *Sh. boydii*, 10^5 cells *S.* Enteritidis and *E. coli*, and 10^6 cells *L. monocytogenes*. This detection limit level observed in this work was less sensitive than that of the microarray sensitivity reported by others. Combination of m-PCR-DNA microarray method detected by fluorescence signal for detection of *E. coli* O157:H7, *S. enterica*, *L. monocytogenes* and *Camp. jejuni* was 10^{-4} ng of each genomic DNA (Suo et al., 2010) and for detection of *V. cholera* was 10^{-2} ng (Kim et al., 2010). Although high sensitivity was found from microarray using fluorescence for signal detection but they need expensive and complicated equipments for signal detection and data analysis. Moreover, more than 5 target bacteria could be detected by our combined methods of m-PCR-oligonucleotide array at as low concentration level as 10 ng of each genomic DNA in pure cultures. With this reasons, multiple target bacteria could be simultaneously detected in single reaction of m-PCR followed by oligonucleotide array with high accuracy and save reactions cost. For sensitivity of combination of conventional PCR-oligonucleotide array in pure culture, combination of conventional PCR and oligonucleotide array was able to detect 10^5 cells of all 4 target bacteria and 10^2-10^3 cells of *E. coli*, *S.* Enteritidis and *Sh. boydii*. The detection

limit for detection of *E. coli*, *S.* Enteritidis and *Sh. boydii* was similar to previously reported of using fluorescence detection system and other colorimetric methods which reported the detection limit of only single target bacteria detection (10^2-10^3 CFU/ml) (Wang et al., 2007; Bai et al., 2010; Quiñones et al., 2011) or multiple target bacterial detection 10^3 CFU/ml (Suo et al., 2010). The detection sensitivity using mixture of genomic DNA from 4 target bacteria ranging from 10-0.001 ng/µl as templates was compared with number of bacterial cells. Results showed that the detection limit for all 4 target bacteria was 0.1 ng of each genomic DNA and for 3 target bacteria including *E. coli*, *L. monocytogenes*, and *Sh. boydii* was 0.01 ng of each genomic DNA. These results demonstrated that the amount of genomic DNA of Gram-positive bacteria, *L. monocytogenes*, was lost during the DNA extraction step. Therefore, yield of the genomic DNA extracted from *L. monocytogenes* was lower than that of other Gram-negative bacteria at the same level of cell concentration. By this reasons, the detection of *L. monocytogenes* in real sample were carried out using total genomic DNA extracted from Fraser culture as template alone for amplification of *prf*A gene by m-PCR or conventional PCR. Compared with m-PCR-oligonucleotide array, amplification of specific gene with single primer by conventional PCR prior to apply to oligonucleotide array was more sensitive than that by m-PCR.

In raw meat sample, pathogens are often present in very low level (1-2 cells/25 g food) in a relatively high background of microflora (Suo et al., 2010). Therefore, enrichment steps are very important to increase the target bacterial cell concentration prior to oligonucleotide array detection. Performing an enrichment step on a suspect food sample adds time to the overall detection regime and precludes the ability to

enumerate the original density of the target pathogen. However, enrichment is necessary and, of course, extremely common for target bacteria detection.

Our preliminary investigation found that the universal BPW broth could be used for simultaneously grow all of the target bacteria. After 24 h cultivation of 4 target bacteria in 225 ml BPW (without food sample), *S.* Enteritidis, *E. coli*, and *Sh. boydii* were detected using oligonucleotide array at initial cell concentration of 1 cell of each but not for *L. monocytogenes*. This result agree with the results obtained by Jofŕe and colleagues in 2005 who reported that enrichment of the samples only in BPW produced poor detection of *L. monocytogenes* due to the major growth of *Salmonella* in this broth. By this reason, separate enrichment of two bacterial groups in BPW for enrichment of *Salmonella*, *E. coli*, *Shigella* and Half-Fraser broth (HF) for enrichment of *L. monocytogenes* were preformed prior to total genomic DNA extraction. However some fresh chicken meat samples contained *S.* Enteritidis, *Sh. boydii* and *L. monocytogenes* at very low initial of cell contamination, pre-enriched for 24 h in BPW and HF was not efficient enough for detection of these pathogens by both conventional culture and oligonucleotide array because of high bacterial background. Therefore, to avoid false negative results from the initial contamination at low level, primary pre-enrichment including BPW, HF, Shigella broth and secondary enrichment including, RV broth, TT broth and Fraser broth (United States Food and Drug Administration, 1998) were used.

In the application part, condition of m-PCR used for amplification of target genomic DNA in food samples and pure culture were different. Total genomic DNA extracted from enrichment cultures contained both target and non-target bacteria of high background of microflora. Thus the templates were different from mixture of

genomic DNA extracted from pure culture of only target bacteria. There is a possibility that the presence of the non-target DNAs may interfere with the amplification and/or hybridization of the target DNAs and hence affect the detection sensitivity (Kim et al., 2010). Therefore, optimization of each primer for the amplification of several target bacteria from food samples was necessary. This developed protocol could simultaneously detected 3 target bacteria from fresh chicken meat samples. However, *Shigella* could not be detected from all spiked samples using neither oligonucleotide array nor conventional culture. This problem might be due to the less sensitive from m-PCR amplification. In m-PCR, mixture of several primers sets leads to poor amplification efficiency (Chiang et al., 2006). To avoid this problem, all target genes were amplified from mixed genomic DNA templates by conventional PCR. The mixtures of genomic DNA obtained from the mixture of each enrichment culture were used as template for individually amplified with each specific primer. The result showed that the expected sized of *ipa*H gene amplicon were observed in 5 spiked samples with more than 3 cells of *Sh. boydii* in 25 g samples. This result indicated that amplification of target genomic DNA templates in food sample using m-PCR was less sensitive than that using conventional PCR with single primer set. Thus, to increase the specificity and sensitivity of m-PCR–DNA microarray for multiple pathogen detection, number of genes which can be used for m-PCR in a single tube without sacrificing the sensitivity of hybridization to the microarray should be determined (Kim et al., 2010).

In this works, the 16S rDNA PCR product was observed in agarose gel electrophoresis especially in the amplification of genomic DNA extracted from enrichment culture. However, probes targeted to 16S rRNA gene showed very weak

signal in most sensitivity and food samples application tests (Figure 4.19). This result indicated that the universal primer which can amplified all bacterial genomic DNA templates generated the mixed PCR product which could be observed from agarose gel but was not enough for hybridization with their target probes. In food sample application, total genomic DNA from high background microflora were also amplified by this primer. Thus the 16S rDNA PCR products could contain mixed amplicons of target and non-target bacteria. However, the PCR product obtained from these non-target bacteria showed no cross-reactivity with any probes on the array. Small amount of 16S rDNA PCR product obtained from the target bacteria amplification was not enough for hybridization signal with their specific probes to be observed. Negative signal were found on probes targeted to 16S rRNA gene. But strong signal can be seen using the other gene specific amplification and probe detection. Moreover, weak hybridization signal were observed from positive control (P and 16S) of some blots (Figure 4.4 and Figure 4.6). Since positive control for DNA hybridization process includes 16S rDNA forward primers and DIG-labeled control DNA were kept at -20^{o}C for over two month prior to use as DNA probes and freeze-thaw for several times. Thus it was possible that the efficiency of DNA hybridization might be low because of the degradation of DNA probes. The research finding indicated that quality of DNA probes have strong effect on the DNA hybridization efficiency. Therefore, to avoid repeated freezing and thawing, DNA probes-stock solutions should be aliquoted into smaller volumes.

All results showed in this investigation demonstrated that the 4 target bacteria could be simultaneously detected from fresh chicken meat samples in single array using combination of conventional PCR and oligonucleotide array. By these results,

the oligonucleotide array cost is reduced and easy for screening large number of samples. Moreover, at very low number of cell inoculation of *L. monocytogenes* and *Sh. boydii* in some samples, no PCR product could be detected on agarose gel but positive signal could be observed from the probes on the array. This result confirmed again that the oligonucleotide array is able to increase the detectability compared to the PCR or m-PCR methods.

4.5 Conclusions

In conclusion, this research have developed an oligonucleotide array based method for simultaneous detection of the 3 foodborne pathogens (*Salmonella* spp., *Shigella* spp., and *L. monocytogenes*) and 1 microbial food safety indicator (*E. coli*). At present, oligonucleotide array were combined with target bacterial enrichment and DNA amplification by m-PCR or conventional PCR for screening of 4 common bacteria in fresh chicken meat. The contamination of *Salmonella*, *E. coli*, *Sh. boydii* (at least 3 cells) and *L. monocytogenes* (at least 10 cells) could be detected in 25 g of sample after 24 or 48 h enrichment by combination of conventional PCR and oligonucleotide array. Although the time of multiple pathogen detection by this protocol needs additional 10-15 h for labeling, hybridization and signal detection compared with conventional PCR method, but the analysis time is shorter, more sensitive and easier than that of traditional cultivation approaches. The important advantages of this developed protocol are that it is simpler, more sensitive, higher accuracy and it is sequence-based detection of target genes compared with conventional PCR and m-PCR method. Moreover, this system does not needs any

expensive and special equipment for microarray construction or detection. Thus only a general molecular laboratory is enough for accommodating this developed protocol.

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CHAPTER V

CONCLUSION

This investigation focused on the development of multiple foodborne pathogen detection method in fresh chicken meat by oligonucleotide array hybridization technique. Easy systems for hybridization signal detection using immunological chromogenic reaction which can be observed by naked eyes were performed.

Isolation and characterization of foodborne pathogens and food safety indicators include *Campylobacter* spp., *Clostridium perfringens*, *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* from chicken intestines were attempted. Only *E. coli, Cl. perfringens,* and *Salmonella* spp. were found in this investigation. Characterization of the isolated bacteria were done and found that only minor differences in the biochemical characteristics especially carbohydrate utilization and gelatin hydrolysis were frond from each isolate. The target and non-target bacterial isolates with diverse physiological characteristic were used as the tested organisms for the rapid methods development to increase the specificity of the detection methods. Thus, all primers and probes from this research were tested for the specificity using references and isolated bacterial strains found in enrichment culture.

For optimization of the oligonucleotide array based methods, the 16S rRNA and *prf*A specific gene (specific for *L. monocytogenes*) were selected as model. Target regions labeling by post-PCR labeling method was suitable for probes selection. The

optimum concentration of the oligonucleotide probes was found to be 200 pmol. The detection of foodborne pathogen including *E. coli*, *Cl. perfringens*, *L. monocytogenes*, *Staph. aureus*, and *Salmonella* spp. by oligonucleotide array targeted to the 16S rRNA gene were successfully used for the differentiation of target bacteria in the genus levels. However, cross-reaction of some of the 16S rDNA probes of *E. coli*, *Salmonella* spp., and *L. monocytogenes* were found in *Shigella* spp. and non-target bacteria in the enrichment culture. Thus, m-PCR or conventional PCR were performed to simultaneous amplify species specific genes including *usp*A, *fim*Y, *ipa*H, and *prf*A of *E. coli*, *Salmonella* spp., *Shigella* spp. and *L. monocytogenes* prior to hybridize with their specific probes on the array. Efficiency, specificity and detection limit of m-PCR or conventional PCR couples with the oligonucleotide array for multiple target bacteria detection were evaluated in pure culture and in fresh chicken meat samples. After 24 or 48 h enrichment of each target bacteria from chicken meat samples, the m-PCR or conventional PCR combined with oligonucleotide array could be successfully applied to detect 3 target bacteria including *E. coli*, *L. monocytogenes*, and *Salmonella* sp. or all 4 target bacteria including *E. coli*, *L. monocytogenes*, *Salmonella* sp. and *Sh. boydii* from fresh chicken meat samples, respectively. Application of these combined methods including m-PCR-oligonucleotide and conventional PCR-oligonucleotide array indicated that analysis time is shorter, more sensitive and easier than that of traditional cultivation approaches. Thus identification system in this research could be used as a rapid alternative method to the biochemical confirmation.

APPENDIX

APPENDIX I

MEDIA AND CHEMICAL PREPARATIONS

1. Media for bacterial isolation (M1)

1.1 Buffer Peptone Water (BPW) (United States Food and Drug Administration, 1998)

BPW ingredients (g/l)

Peptone	10.0
Sodium chloride	5.0
Disodium phosphate (Na_2HPO_4)	3.5
Potassium dihydrogen phosphate (KH_2PO_4)	1.5

pH 7.2 ± 0.2

Dissolve ingredients in 1 liter distilled water. Dispense 225 ml of medium into 500 ml Erlenmeyer flasks and autoclave at 121°C for 15 min.

1.2 Lactose broth (LB) (United States Food and Drug Administration, 1998)

Lactose broth ingredients (g/l)

Beef extract	3
Peptone	5

Lactose	5

Final pH, 6.9 ± 0.2.

Dissolve ingredients in 1 liter distilled water. Dispense 225 ml of medium into 500 ml Erlenmeyer flasks and autoclave at 121°C for 15 min.

1.3 Laurul tryptose broth (LST) (United States Food and Drug Administration, 1998)

Laurul tryptose broth ingredients (g/l)

Tryptose	20
Lactose	5
Dipotassium phosphate (K_2HPO_4)	2.75
Potassium dihydrogen phosphate (KH_2PO_4)	2.75
Sodium chloride	5
Sodium lauryl sulfate	0.1

pH 6.8 $\pm$ 0.2

Dissolve ingredients in 1 liter distilled water. Dispense 10 ml of medium into 16 x 150 mm tubes containing inverted 6 x 30 mm Durham tubes and autoclave at 121°C for 15 min.

1.4 Peptone dilution fluid (United States Food and Drug Administration, 1998)

Peptone dilution fluid ingredients (g/l)	
Peptone	10
	Final pH, 7.0

Dissolve ingredients in 1 liter distilled water. Dispense 9 ml of medium into 16 x 150 mm screw-cap tubes and autoclave at 121°C for 15 min.

1.5 Shigella broth (United States Food and Drug Administration, 1998)

Shigella broth based ingredients (g/l)	
Tryptone	20
Dipotassium phosphate (K_2HPO_4)	2
Potassium dihydrogen phosphate (KH_2PO_4)	2
Sodium chloride	5
Glucose	1
Tween 80	1.5 ml
	pH, 7.0 ± 0.2.

Dissolve all ingredients in 1 liter distilled water and autoclave at 121°C for 15 min. Novobiocin (OXIOD) are dissolved in sterile water and added into the sterile medium at final concentration of 3.0 µg/ml.

1.6 Trypticase soy agar (TSA)

Trypticase soy agar ingredients (g/l)	
Tryptone	15
Proteose peptone	5
Sodium chloride	15
Agar	15
	pH, 7.0 ± 0.2.

Dissolve all ingredients in 1 liter distilled water and autoclave at 121°C for 15 min. Cool the medium to 65^{o}C. Mix well and pour into sterile Petri dishes.

1.7 Trypticase soy broth (TSB)

Trypticase soy broth ingredients (g/l)	
Tryptone	15
Proteose peptone	5
Sodium chloride	15
	pH, 7.0 ± 0.2.

Dissolve all ingredients in 1 liter distilled water and autoclave at 121°C for 15 min.

2. Media for biochemical test (M2)

2.1 Gelatin hydrolysis medium (Cappuccino and Sherman, 1999**)**

Gelatin hydrolysis medium ingredients (g/l)	
Gelatin	120

Dissolve gelatin in 1 liter of nutrient broth. Heat a medium with agitation to dissolve gelatin. Dispense 10 ml of medium into 16 x 150 mm tubes and autoclave at 121°C for 15 min.

2.2 Iron Milk Medium (United States Food and Drug Administration, 1998**)**

Iron Milk Medium ingredients (g/l)

Ferrous sulfate.$7H_2O$ ($FeSO_4.7H_2O$)	1
Fresh whole milk	1 liter
Distilled water	50 ml

Dissolve ferrous sulfate in 50 ml distilled water and slowly add to 1 liter milk. Mix medium with magnetic stirrer. Dispense 11 ml of medium into 16 x 150 mm culture tubes and autoclave 12 min at 118°C.

2.3 Lactose-Gelatin Medium (United States Food and Drug Administration, 1998**)**

Lactose-Gelatin Medium ingredients (g/l)

Tryptose	15
Yeast extract	10
Lactose	10
Phenol red (1% solution in 95% ethanol)	5.0 ml
Gelatin	120

pH to 7.5 ± 0.2

Dissolve tryptose, yeast extract, and lactose in 400 ml water. Suspend gelatin in 600 ml water and heat at 50-60°C with agitation to dissolve. Mix 2 solutions and adjust pH to 7.5 ± 0.2. Add phenol red and mix. Dispense 10 ml of medium into 16 x 150 mm screw-cap tubes and autoclave at 121°C for 10 min. If not used within 8 h, deaerate by heating at 50-70°C for 2-3 h before use.

2.4 Motility-Nitrate Medium, Buffered (United States Food and Drug Administration, 1998)

Motility-Nitrate Medium, Buffered ingredients (g/l)

Beef extract	3
Peptone	5
Potassium nitrate (KNO_3)	1
Disodium phosphonate (Na_2HPO_3)	2.5
Galactose	5
Glycerin	5 ml

pH to 7.3 ± 0.1.

Dissolve all ingredients in 1 liter of distilled water and heat to dissolve. Dispense 10 of medium into 16 x 150 mm tubes and autoclave at 121°C for 15 min. If not used within 4 h, heat 10 min in boiling water or flowing steam. Chill in cold water.

2.5 Motility Test Medium (Semi-solid) for *L. monocytogenes* test (United States Food and Drug Administration, 1998**)**

Motility Test Medium ingredient (g/l)	
Beef extract	3
Peptone or gelysate	10
Sodium chloride	5
Agar	4

pH, 7.4 ± 0.2.

Dissolve all ingredients in 1 liter distilled water. Heat a medium with agitation to dissolve agar. Dispense 8 ml of medium into 16 x 150 screw-cap tubes and autoclave at 121°C for 15 min.

2.6 MR-VP broth (United States Food and Drug Administration, 1998**)**

MR-VP broth ingredient (g/l)	
Peptone	5
Glucose	5
Dipotassium phosphate (K_2HPO_4)	5

pH, 7.5 ± 0.2.

Dissolve all ingredients in 1 liter distilled water. Dispense 5 ml of medium into 13 x 100 mm test tubes and autoclave at 121°C for 15 min.

2.7 Purple Carbohydrate Broth based, 0.5% carbohydrtae (United States Food and Drug Administration, 1998**)**

Purple Carbohydrate Broth based ingredients (g/l)

Proteose peptone	10
Beef extract	1
Sodium chloride	5
Bromcresol purple	0.02

Final pH, 6.8-7

Dissolve all ingredients, omitting carbohydrate, in 800 ml distilled water with heat and occasional agitation. Dispense 2.0 ml of medium into 13 x 100 mm test tubes containing inverted Durham tubes and autoclave at 118°C for 15 min. Dissolve carbohydrate in distilled water at final concentration of 2.5% and sterilize by passing solution through bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to less than 45°C. Shake gently to mix.

Carbohydrate solution; 2.5% dextrose, maltose, rhamnose, mannitol, xylose, myo-inositol, lactose.

2.8 SIM agar (Cappuccino and Sherman, 1999)

SIM agar ingredients (g/l)

Peptone	30
Beef extract	3
Ferrous ammonium sulfate	0.2

$(Fe(NH_4)_2SO_4)_2\ 6H_2O)$	
Sodium thiosulfate	0.025
$(Na_2S_2O_3)$	
Agar	3

pH, 7.3

Dissolve all ingredients in 1 liter distilled water. Heat a medium with agitation to dissolve agar. Dispense 8 ml of medium into 13 x 100 screw-cap tubes and autoclave at 121°C for 15 min.

2.9 Simmons citrate agar slant (Cappuccino and Sherman, 1999)

Simmons citrate agar ingredients (g/l)

Ammonium dihydrogen phosphate	1
$(NH_4H_2PO_4)$	
Dipotassium phosphate	1
(K_2HPO_4)	
Sodium chloride	5
Sodium citrate	2
$(NaH_2C_6H_5O_7)$	
Magnesium sulfate	0.2
$(MgSO_4 \cdot 7H_2O)$	
Agar	15
Bromothymol blue	0.08

pH, 6.9

Dissolve all ingredients in 1 liter of distilled water. Heat a medium with agitation to dissolve agar. Dispense 10 ml of medium into 16 x 150 mm test tubes and autoclave at 121°C for 15 min. While the medium is hot, tilt the tube. Medium in the tubes will be slanted. Allow the medium to harden in this position.

2.10 Trypticase nitrate broth (Cappuccino and Sherman, 1999**)**

Trypticase nitrate broth ingredients (g/l)

Trypticase	20
Disodium phosphate (Na_2HPO_4)	2
Dextrose	1
Agar	1
Potassium nitrate (KNO_3)	1

pH 7.2

Dissolve all ingredients in 1 liter of distilled water. Heat a medium with agitation to dissolve agar. Dispense 10 ml of medium into 16 x 150 mm test tubes and autoclave at 121°C for 15 min.

2.11 Urea Broth (United States Food and Drug Administration, 1998**)**

Urea Broth ingredients (g/l)

Urea	20
Yeast extract	0.1
Disodium Hydrogen Phosphate	9.5

(Na_2HPO_4)	
Potassium Dihydrogen Phosphate	9.1
(KH_2PO_4)	
Phenol red	0.01

pH, 6.8 ± 0.2.

Dissolve all ingredients in 1 liter of distilled water. **DO NOT HEAT.** Sterilize by filtration through 0.45 µm membrane. Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes.

3. Chemical for bacterial isolation (C3)

3.1 Butterfield's phosphate-buffered water (United States Food and Drug Administration, 1998)

Butterfield's phosphate-buffered water compound	
Potassium Dihydrogen Phosphate (KH_2PO_4)	34 g
Distilled water	500 ml
	pH 7.2

Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 liter with distilled water. Sterilize at 121°C for 15 min. Store in refrigerator.

4. Chemical for biochemical test (C4)

4.1 Indole test (United States Food and Drug Administration, 1998)

Kovacs' reagent

p-dimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
HCl (concentrated)	25 ml

Dissolve *p*-dimethylaminobenzaldehyde in amyl alcohol. Slowly add HCl into a solution. Store the Kovacs' reagent at 4°C.

4.2 Methyl red- reactive compound test (United States Food and Drug Administration, 1998)

Methyl red- indicator compound

Methyl red	0.10 g
Ethanol, 95%	300 ml
Distilled water to make	500 ml

Dissolve methyl red in 300 ml ethanol. Bring volume to 500 ml with distilled water.

Store the methyl red- indicator compound at room temperature.

4.3 Nitrate reduction test (United States Food and Drug Administration, 1998)

Solution A (Sulfanilic acid reagent)

Sulfanilic acid	1 g

5 N acetic acid	125 ml

Solution B (N-(l-naphthyl)ethylenediamine reagent)

N-(l-naphthyl)ethylenediamine-dihydrochloride	0.25 g
5 N acetic acid	200 ml

Solution C (α-Naphthol reagent)

α-Naphthol	1 g
5 N acetic acid	200 ml

To prepare 5 N acetic acid, add 28.75 ml glacial acetic acid to 71.25 ml distilled water. Store the reagents in glass-stopped brown bottles.

4.4 Voges-Proskauer (VP) (United States Food and Drug Administration, 1998)

Barritt's reagent A

α-Naphthol	5 g
Alcohol (absolute)	100 ml

Barritt's reagent B

Potassium hydroxide (KOH)	40 g
Distilled water to make	100 ml

APPENDIX II

BIOCHEMICAL ANALYSIS

1. Catalase test (Cappuccino and Sherman, 1999)

Procedures

On a glass slide, drop one drop of the 3% hydrogen peroxide. Single colony of the tested organism grown on TSA (Appendix I, M1.6) or TSC agar for 24 h was picked by sterile toothpick and smeared in a drop of 3% hydrogen peroxide on glass slide. Each hydrogen peroxide drop was examined for the presence or absence of bubbling or foaming.

2. Carbohydrate fermentation test (United States Food and Drug Administration, 1998)

Procedures

The tested organism were grown in TSB (Appendix I, M1.7) for 24 h. Using sterile technique, each experimental organism from TSB culture was inoculated into appropriately labeled medium containing each sugar by loop inoculation. During this step take care not to shake the fermentation tube containing purple carbohydrate broth (Appendix I, M2.7). All tubes were incubated for 24 h at 37^{o}C for *E. coli*, *Salmonella* spp. and 72 h for *L. monocytogenes*.

3. **Gelatin hydrolysis** (Cappuccino and Sherman, 1999)

Procedures

The tested organism were grown in TSB (Appendix I, M1.7) for 24 h. Using sterile technique, inoculated each experimental organism culture in its appropriately labeled gelatin hydrolysis medium (Appendix I, M2.1) deep tube by a stab inoculation. The culture was incubated at 37^{o}C for 48 h. The cultures were placed in refrigerator at 4^{o}C for 30 min. Cultures that remain liquefies produce gelatinase and demonstrated rapid gelatin hydrolysis. Re-incubated all solidified culture for an additional 5 days at 37^{o}C. The cultures were placed in refrigerator at 4^{o}C for 30 min and observed for liquefaction.

4. **Gelatin liquefaction and lactose fermentation test** (United States Food and Drug Administration, 1998)

Procedures

Lactose-gelatin media (Appendix I, M2.3) were inoculated by stab with a loopfuls of *C. perfringens* culture in fluid thioglycollate medium (Himedia). Lactose-gelatin medium cultures were incubated at 35°C for 24 h. Cultures for gas production and color change from red to yellow were examined, which indicates acid production. Tubes were chilled at 4°C for 1 h and examined for gelatin liquefaction. If medium gels, incubated an additional 24 h at 35°C and examined for gelatin liquefaction.

5. **Hemolysis test** (United States Food and Drug Administration, 1998)

Procedures

Listeria monocytogenes posses beta-hemolytic activity on sheep blood agar plates but often produce only narrow zones of hemolysis that frequently do not extend much

beyond the edge of the colonies. Fresh single colonies of *Listeria* sp. on TSA (Appendix I, M1.6) were streaked on sheep blood agar (OXIOD) and incubated at 37^{o}C for 24 h. Then observed for the hemolytic activity.

6. IMViC test (Cappuccino and Sherman, 1999)

Principle

IMViC stands for four main tests. “I” is for indole; “M” is for methyl red; “V” is for Voges-Proskauer, and “C” is for citrate. IMVic tests are a series of test used for differentiation of Enterobacteriaceae group base on their biochemical properties and enzymatic reactions in the presence of specific substrate.

a. Indole production test

Procedures

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 37^{o}C for 24 to 48 h. The cultures were inoculated into deep tube containing SIM agar (Appendix I, M2.8) by mean of a stab inoculation. SIM cultures were incubated at 35^{o}C for 24 to 48 h. Ten drops of Kovac’s reagent (Appendix I, C4.1) were added into all deep tube cultures and agitate the cultures gently. The color of the reagent layer in each culture was examined.

b. Methyl red reactive compound test

Procedures

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 37^{o}C for 24 to 48 h. The cultures were inoculated into MR-VP broth (Appendix I, M2.6) by means of a loop inoculation and incubated at 37^{o}C for 24 to 48 h. One-third of each culture was transferred into an empty test tube and then set aside for Voges-Proskauer

test. Five drops of the methyl red indicator (Appendix I, C4.2) were added to the remaining aliquot of each culture.

c. Voges-proskauer (VP) reaction

Procedures

The aliquot of each MR-VP broth (Appendix I, M2.6) culture separated during methyl red reactive compound test were added with Barritt's reagent A (Appendix I, C4.4) and shaken. Immediately 10 drops of Barritt's reagent B (Appendix I, C4.4) were added and shaken the culture. The culture were re-shaken every 3 to 4 min. The color of the cultures 15 min after the addition of Barritt's reagent were examined and recorded.

d. Citrate test

Procedures

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 37^{o}C for 24 to 48 h. The cultures were inoculated into Simmons citrate agar slants (Appendix I, M2.9) by means of a stab and streaked and incubated at 37^{o}C for 24 to 48 h. All agar slat cultures were examined for the presence or absence of growth and coloration of the medium.

7. Motility and H_2S production test (Cappuccino and Sherman, 1999)

Procedures

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 32^{o}C for 24 to 48 h.

a. *E. coli* and *Salmonella* spp.

The TSB cultures were inoculated into deep tube containing SIM agar (Appendix I, M2.8) by mean of a stab inoculation and incubated at 35^{o}C for 24 to 48

h. All SIM cultures were examined for the presence or absence of black coloration along the line of the stab inoculation and presence or absence of motility.

b. *Listeria* spp.

Procedures

For H_2S production test, the TSB cultures were inoculated into a deep tube containing SIM agar (Appendix I, M2.8) by mean of a stab inoculation and incubated at 35°C for 24 to 48 h. All SIM cultures were examined for the presence or absence of black coloration along the line of the stab inoculation.

For motility test, MTM provides the best defined umbrellas. The cultures of *L. monocytogenes* in TSB were inoculated into deep tube containing MTM agar (Appendix I, M2.5) and incubated at room temperature (25°C) for at least 48 h. Results were observed and recorded daily within 7 days of incubation. In tubes with motile characteristic of umbrella shape were recorded as positive.

c. *C. perfringens*

Procedures

Single colony of *C. perfringens* were transferred to fluid thioglycollate medium (Himedia) and incubated under anaerobic condition at 37°C. A loopfuls of thioglycollate medium culture was inoculated into motility-nitrate buffered medium (Appendix I, M2.4). The cultures were incubated at 35°C for 24 h. The presences or absences of motility along the line of the stab inoculation were observed. The cultures were further tested for nitrate reduction.

8. Nitrate reduction test (United States Food and Drug Administration, 1998)

Procedures

a. *E. coli* and *Salmonella* spp.

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 37^{o}C for 24 to 48 h. Each culture was inoculated into tube containing trypticase nitrate broth (Appendix I, M2.10) by mean of a loop inoculation. The cultures were incubated at 37^{o}C for 24 to 48 h. All cultures were added with five drops of solution A and five drops of solution B (Appendix I, C4.3). A red coloration developed in each of the culture was recorded as positive results. If no color developed, a few grains of powdered zinc metal were added. No red color indicated that nitrates were completely reduced, positive results were recorded.

b. *C. perfringens*

A culture of *C. perfringens* in motility-nitrate buffered (Appendix I, M2.4) were tested for nitrate reduction. Solution A 0.5 ml and solution B 0.2 ml (Appendix I, C4.3) was added into motility-nitrate buffered culture. Results with violet color developed within 5 min indicated that presence of nitrites has occurred, positive results were recorded. If no color developed, a few grains of powdered zinc metal were added. No violet color indicated that nitrates were completely reduced, positive results were recorded.

9. Oxidase test (Cappuccino and Sherman, 1999)

Procedures

A fresh culture (18 to 24 h) of bacteria was grown on nutrient agar using the streak plate method**.** One or two drops of oxidase reagent (tetramethyl-p-phenylenediamine) were added to the colonies on plate. Color of the colonies was observed after addition of the solution. Colors of colonies changed to violet were recorded as positive.

10. Stormy fermentation (United States Food and Drug Administration, 1998)

Procedures

One ml of actively grown fluid thioglycollate (Himedia) culture of *C. perfringens* was transferred to modified iron-milk medium (Appendix I, M2.2). Culture medium was incubated at 46°C for 5 h but checked for stormy fermentation after 2 h. After 2 h incubation, checked hourly for "stormy fermentation". Stormy fermentation reaction has been characterized by rapid coagulation of milk followed by fracturing of curd into spongy mass which usually rises above the medium surface.

11. Urea hydrolysis (United States Food and Drug Administration, 1998)

Procedures

Experimental microorganism was grown in TSB (Appendix I, M1.7) at 37^{o}C for 24 to 48 h. A loopful of TSB culture was inoculated into tube containing urea broth (Appendix I, M2.11). The urea broth cultures were incubated at 37^{o}C for 24 to 48 h. All urea broth cultures were examined for color change.

BIOGRAPHY

Chanida Kupradit was born in 1980 in Nakhnon Ratchasima, Thailand. In March 1999, she graduated with a high school diploma majoring in Science from Boonwatthana Secondary School, Nakhon Ratchasima, Thailand. In May 2003, she graduated with a Bachelor degree of Science majoring in Biotechnology from Khon Kaen University (KKU), Khon Kaen, Thailand. In March 2007, she graduated with a Master degree of Science from the School of Biotechnology, Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. She has work as a research assistant at Siriraj hospital for a short time before entering the Ph.D. program in Biotechnology at SUT. She obtained a Ph.D. scholarship from the commission of higher education (CHE), Thailand. During her Ph.D. works, she has presented two poster presentations and one national publication. She is now in the process of preparing three manuscripts to submit to two international journals and one national journal.