MAGNETIC NANOPARTICLE BASED PCR ASSAY FOR DETECTION OF *CAMPYLOBACTER* SPECIES IN FOOD SAMPLES AND DIELECTROPHORETIC IMPEDANCE MEASUREMENT FOR DNA DETECTION

WUTTICHOTE JANSAENTO

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ABSTRACT

Campylobacter species are major foodborne pathogens, causing bacterial gastroenteritis worldwide. Poultry are considered to be important reservoirs of *Campylobacter* transmission and campylobacteriosis commonly relates to the consumption of undercooked chicken products. This study successfully developed a magneto-PCR-enzyme linked gene assay for the detection of thermophilic *Campylobacter* from chicken skin. The forward primers immobilized on magnetic nanoparticles captured *Campylobacter* DNA to be amplified by PCR reaction and the PCR products were detected by streptavidin-horseradish peroxidase and its substrate. The method demonstrated 100% specificity and high sensitivity with one picogram detection of *Campylobacter* DNA. All positive results derived from the detection in naturally contaminated samples were in accordance with the results analyzed by a conventional cultural method and PCR assay. In this study, the dielectrophoretic impedance measurement (DEPIM) method has been used for the detection of PCR and LAMP products by measuring an increment of the conductance. Dielectrophoresis is a motion of the polarized particles in spatially non-uniform electric fields. The DEP-trapping process of DNA, generated from PCR or LAMP products, was not visualized by a fluorescence microscopy. However, in an AC electric field of 150 kHz, the conductance increased when applying the undiluted PCR product of 187 base pairs. After optimizing conditions for detecting LAMP product, only undiluted and ten-fold diluted LAMP products significantly demonstrated the conductance increment. This preliminary study showed the possibility of applying The DEPIM method for detection of PCR and LAMP products.

KEY WORDS: MAGNETIC NANOPARTICLE/ CHICKEN SKIN/ PCR/LAMP/ THERMOPHILIC *CAMPYLOBACTER* / DIELECTROPHORESIS/

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การพัฒนาวิธี PCR โดยใช้ magnetic nanoparticle เพื่อตรวจหา *Campylobacter* species ในตัวอย่างอาหารและการใช้เทคนิค dielectrophoretic impedance ในการตรวจวัดดีเอ็นเอ

MAGNETIC NANOPARTICLE BASED PCR ASSAY FOR DETECTION OF *CAMPYLOBACTER* SPECIES IN FOOD SAMPLES AND DIELECTROPHORETIC IMPEDANCE MEASUREMENT FOR DNA DETECTION

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บทคัดย่อ

เชื้อ *Campylobacter* เป็นแบคทีเรียก่อโรคที่สำคัญที่ติดต่อได้ทางอาหารและพบการก่อโรคลำไส้อักเสบได้ทั่วโลก สัตว์ปีกโดยเฉพาะไก่จัดเป็นแหล่งสะสมเชื้อตามธรรมชาติที่สำคัญและโรคติดเชื้อที่เกิดขึ้นมักเกี่ยวข้องกับการรับประทานเนื้อไก่และผลิตภัณฑ์จากไก่ที่ปรุงไม่สุก ผู้วิจัยได้พัฒนาวิธี magneto-PCR-enzyme linked gene เพื่อตรวจหาเชื้อ *Campylobacter* จากตัวอย่างหนังไก่ขึ้น โดย forward primer ไปติดกับอนุภาคแม่เหล็กขนาดนาโนเพื่อจับกับสายพันธุกรรมของเชื้อนี้และนำไปทำปฏิกิริยา PCR เพื่อเพิ่มจำนวนสายพันธุกรรม ทำการตรวจหาผลผลิต PCR โดยใช้ streptavidin-horseradish peroxidase ย่อยสารตั้งต้นที่เติมลงไปและเมื่อมีการย่อยเกิดขึ้นสามารถวัดการดูดกลืนแสงได้ ซึ่งวิธีการดังกล่าวมีความจำเพาะเป็น 100 เปอร์เซ็นต์และมีความไวสูงโดยตรวจได้ในปริมาณสารพันธุกรรมของ *Campylobacter* ต่ำที่สุดได้ระดับ 1 พิโคกรัม เมื่อนำวิธีนี้ไปทำการทดสอบกับตัวอย่างหนังไก่ที่เป็นบวก พบว่ามีความสอดคล้องกับผลบวกที่ได้จากการทำการวิเคราะห์ด้วยวิธีการเพาะเลี้ยงมาตรฐานและวิธี PCR ปกติ นอกจากนี้ผู้วิจัยได้นำเทคนิค dielectrophoresis ร่วมกับการวัดค่าความเหนี่ยวนำไฟฟ้ามาใช้ในการตรวจหาผลผลิตจากปฏิกิริยา PCR และ LAMP ซึ่งเทคนิค dielectrophoresis เป็นการเคลื่อนที่ของวัสดุที่มีขั้วประจุในสนามไฟฟ้าที่ไม่เป็นเนื้อเดียวกัน การศึกษานี้ไม่สามารถแสดงภาพการจับของสายพันธุกรรมกับขั้วไฟฟ้าได้เมื่อดูภายใต้กล้องจุลทรรศน์ แต่สามารถวัดการเพิ่มขึ้นของค่าความเหนี่ยวนำไฟฟ้าเมื่อใช้ตัวอย่างที่เป็นผลผลิตจาก PCR ขนาด 187 คู่เบส เมื่อใช้กระแสสลับที่ความถี่ 150 kHz ตัวอย่างจากปฏิกิริยา LAMP ทั้งที่ไม่เจือจางและเจือจาง 10 เท่ามีผลเพิ่มค่าความเหนี่ยวนำไฟฟ้าได้ดี งานวิจัยนี้แสดงให้เห็นความเป็นไปได้ที่จะประยุกต์ใช้เทคนิคนี้ในการตรวจหาสารพันธุกรรมจากปฏิกิริยา PCR และ LAMP

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LIST OF ABBREVIATIONS

AC	Alternate current
ABTS	2,2’-azino-di(3-ethylbenzthiazoline-6-sulfonic acid
bp	Base pair(s)
CO_2	Carbon dioxide
cm	Centimeter(s)
cfu	Colony forming units
^{O}C	Degrees Celsius
DEP	Dielectrophoresis
DEPIM	Dielectrophoretic impedance measurement
DC	Direct current
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FISH	Fluorescence *in situ* hybridization
g	Gram(s)
HRP	Horseradish peroxidase
h	Hour(s)
kHz	KiloHertz
LAB	Lactic acid bacteria
L	Liter(s)
pH	-Log hydrogen ion concentration
LAMP	Loop-mediated isothermal amplification
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
µS	Microsievert(s)
mg	Milligram(s)
mL	Milliliter(s)

LIST OF ABBREVIATIONS (cont.)

min	Minute(s)
M	Molar(s)
MNP	Magnetic nanoparticle(s)
OD	Optical density(-ies)
PNP	Peptide nucleic acid
%	Percent
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SA	Streptavidin
V	Voltage
VBNC	Viable but non-culturable

CHAPTER I
INTRODUCTION

Campylobacter species are recognized as major foodborne pathogens causing gastroenteritis both in developed and developing countries. The significant symptoms of the infection include diarrhea, abdominal pain, fever and malaise, however, the disease is usually self-limited within a week (1). Severe complications of post-infection by *C. jejuni* that may occur are reactive arthritis and Guillain-Barré syndrome which is a life-threatening acute paralytic disease. *Campylobacter jejuni*, *C. coli* and *C. lari* are thermotolerant campylobacters that mostly related with *Campylobacter* infections in human. Thermotolerant campylobacters are naturally found in intestinal tracts of domestic and wild animals, particularly poultry that are reported as important sources of the disease transmission in human (2). A high body temperature of birds may be an optimal condition for a growth of thermophilic campylobacters. The transmission of campylobacters is mainly by fecal-oral route via a consumption of contaminated undercooked poultry products, raw milk and surface water (3). In Thailand, isolation rates of *Campylobacter* species from retail poultry were 12-65% depending on types of chicken products and regions of the country (4-7). In addition, the isolation rates from diarrheaic specimens of children less than 5 years old were 12-13% (8, 9) with the highest prevalence at the age of less than 12 months (10). A large number of campylobacters is usually found in stools of the infected human while the low number is persisted in food samples. An infective dose of 800-10,000 *Campylobacter* cells is required to cause an infection, also depending on the bacterial strains and host susceptibility (11).

Standard cultural method according to ISO10272: 2006 for detection of *Campylobacter* spp. in food and animal feeding stuffs requires an enrichment step for for 48 h, colony isolation on selective agar plates for 48 h, and identification by biochemical testing for at least 48 h (12). The process of culture-based method is time-consuming and labor-intensive; therefore, a rapid and reliable method is needed for *Campylobacter* detection. Nucleic acid-based methods have been widely developed

with high sensitivity and high specificity for detection of foodborne pathogens from food samples and clinical specimens. The amplification technique is also useful for detection of *Campylobacter* cells that are in a viable but non-culturable state which could not be detected by the cultural method (13). Many assays based on PCR technology have been reported for *Campylobacter* detection in fecal samples (14, 15), clinical specimens (16, 17), broiler products (18) and environmental samples (19). Some PCR techniques still require gel electrophoresis and ethidium bromide staining to reveal target amplicons while real-time PCR assays are used to detect PCR products simultaneously by an expensive thermal cycle with a fluorescence detector. Loop-mediated isothermal amplification (LAMP) method has been developed for detecting various infectious microorganisms because of its more simplicity and rapidity. A basic principle of LAMP is an autocycling strand displacement DNA synthesis using Bst DNA polymerase in a reaction temperature between 60-65°C. Up to six primers are used to confer high specificity and a turbidity of LAMP product can be judged by naked eyes without running electrophoresis (20). LAMP assays were currently developed for detection of *C. jejuni* and *C. coli* in pure cultures, food and clinical samples (21-24). The aims of most new detection methods are implemented to have short detection period, high sensitivity and high specificity. Some detection methods may be proper for particular type of samples which may not suitable for other types of samples. Food samples have different characteristics from clinical samples. They contain various kinds of bacterial groups, different matrices and complex substances which may interfere the detection system. Sample collection and sample preparation should be considered prior to perform the detection process.

Magnetic nanoparticles (MNPs) have been successfully applied in the assay development for detection of microorganisms and biological moleculeles, such as nucleic acids and proteins because of (25). Magnetic microbeads generally are core-shell structures that have iron oxide as an inorganic core and the shell often consists of functionalized long chain organic ligands or inorganic/organic polymers. Paramagnetic particles tagged with specific antibodies for foodborne pathogens (e.g. *Salmonella, Campylobacter, Escherichia coli, Listeria,* and *Shigella*) have been developed for selective seperation of target microoganisms from the crude samples with magnetic field (26-30). After protocols for immobilizing of primers on magnetic

nanoparticles are successfully developed (31-34) The magnetic-based PCR techniques have been employed for nucleic acid detection with a variety of detection systems such as agarose gel electrophoresis (35), fluorescence (34), electrochemical (36) and atomic force microscopic (31) methods. This thesis applied an enzyme-substrate system for a simple detection of PCR amplicons that attached on the magnetic nanoparticles.

Dielectrophoresis (DEP) is a useful technique for separations of a variety of cells, nanoparticles and biomolecules (37). Dielectrophoretic force is exerted on a polarizable object which its surface charges are induced in the non-uniform electric field. The net force subsequently pushes on the object that having higher polarizability than the surrounding medium towards the region of higher field strength and this phenomenon is called positive DEP (38). DEP has been widely applied for manipulation of DNA such as DNA trapping, stretching, separation, purification, and concentration, both at a nanoscale or a larger scale (39, 40). Dielectrophoretic impedance measurement (DEPIM) has been developed for detection of biological cells by using alternating current (AC) dielectrophoresis and measuring electrical conductance of intact cells captured on microelectrodes (41). The DEPIM was applied to combine with electropermeabilization for higher sensitivity of bacterial and yeast cells detection (42), or antigen-antibody reaction for selective detection of bacterial species (43, 44). It is used also to selective detection of viable bacterial cells (45) and to rapidly quantify oral bacteria with a developed apparatus (46).

Therefore, the objective of this study was to develop PCR technique combined with magnetic nanoparticle and enzyme-substrate detection system for detection of thermophilic *Campylobacter* in pure culture and in natural contaminated chicken skin. Another objective was to apply the DEPIM technique using castle-walled type of microelectrodes for capturing double-stranded DNA that was experimentally generated for PCR and LAMP methods

CHAPTER II
LITERATURES REVIEW

2.1 Characteristics of *Campylobacter*

Campylobacter is known as an importance food borne pathogen among various causative agents of diarrhea and gastroenteritis. Genus *Campylobacter* belongs to delta-epsilon group of proteobacteria comprising of members that are small gram-negative, microaerophilic, curved-to-spiral rods in 1.5-3.5 μm long by 0.2-0.4 μm wide. They have a single polar flagellum at one or both ends of the cell and are non-spore forming. They are motile with a characteristic of corkscrew like movement. They may also be gull or S-shaped while *C. fetus* may appear ribbon-shaped comprising a chain of single curved cells and cells may become coccoid in old cultures. Important bacteria in this genus is *C. jejuni* that has a 1,641,481 base pairs circular chromosome with 30.6% G+C content which is possibly to encode 54 stable RNA species and 1,654 protein molecules (47). The genome sequence of *C. jejuni* has very few repeat sequences, no phage-associated sequences or insertion sequences, and hyper variable sequences. These organisms can colonize in the intestinal tract and oral cavity in human and animals and are associated with abortion and reproductive diseases in animals and with a variety of infections in human (48).

Genus *Campylobacter* was assigned in 1963 that comprised only two species. The taxonomy has dramatically changed at the present. The genus *Campylobacter* belongs to a phylogenetically group referred to as the epsilon division and ribosomal RNA (rRNA) superfamily VI of the class Proteobacteria. The diversity of this group is matched by the diversity of habitats and the wide range of diseases that they may be found and associated (49). Species of *Campylobacter* with natural habitats found and disease associated are as followed: *C. jejuni* subsp. *jejuni* has been isolated form poultry, pigs, cats, dogs, birds, and other animals. *C. coli* can be found in pigs, poultry, sheep, bulls and birds. *C. lari* was found in birds, poultry, other animals, river and sea water. *C. upsaliensis* can be detected in dogs and cats (50).

2.2 Morphology and growth conditions

Campylobacter requires microaerobic conditions consisting of 5% of oxygen, 10% of carbon dioxide and 85% of nitrogen for optimal growth. *C. jejuni* growth at 37°C and 42°C has different lipo-oligo-saccharide (LOS) forms both of human and chicken isolated strains. The rising temperature increases the multiple lower-M_r form contained a β-D-Gal-(1→3)-β-D-GalNAc disaccharide moiety which is consistent with the termini of the GM_1, asialo-GM_1, GD_1, GT_1 and GQ_1 gangliosides while it lacks of GM_1 mimicry. The production of lower-M_r form was dependent on the growth temperature as the production of this form increases from ~5% when growth at 37°C to ~35% when growth at 42°C (51).

Campylobacter can survive in unfavorable conditions by forming a dramatic morphological transformation from an S-shaped rod to a coccoid form, which is called the viable but non-culturable (VBNC) state (52, 53). This characteristic plays an important role in the survival and dissemination of *C. jejuni*. However there are some different properties between coccoid form at low and high temperatures. When incubation at 37°C, there is a progressive degradation of DNA in coccoid form identical to that of S-shaped cells while incubation at 4°C an S-shape was retained for 8 weeks and a few DNA was released into the medium. Low temperature seems to inhibit both coccoid formation and genome degradation which is the most probable candidate for a VBNC form of this pathogen (54). In the VBNC state, campylobacter cells can not grow on cultural media but the cells exhibited resuscitation after passage on experimental animal (55, 56).

C. jejuni growth was inhibited in the presence of lactic acid bacteria (LAB) including *Lactobacillus pentosus* CWBI B78 and *Enterococcus faecium* THT, especially supplementation with a cellulase A complex. Both LAB have antibacterial effect to *C. jejuni* due to *L. pentosus* CWBI B78 produces lactic acid while *E. faecium* THT produces lactic and acetic acids. Antibacterial effect can be observed positively correlation enhancing with the cellulase A concentration. This evidence could be used in planning strategies targeting at the reduction of *Campylobacter* prevalence in poultry production chain which consequently reduces risk of infection (57).

2.3 Epidemiology and transmission

Genus *Campylobacter* contains at least 16 species and 6 subspecies and thermophilic *Campylobacter* species, mainly *C. jejuni*, are major causative agents of bacterial gastroenteritis worldwide (1). The symptoms include inflammation, abdominal pain, fever and diarrhea. The infection is self-limiting with an incubation period of 7-10 days (2). Other than *C, jejuni*, *C. coli* can cause about 10-15% of infectious cases. *C. lari* and *C. upsaliensis* have been occasionally isolated from human infections (58). Infective dose of *Campylobacter* is about 500-10,000 cells and infections also depend on bacterial strains and host immunity (59, 60). Postinfection complications of *C. jejuni* include reactive arthritis and Guillain-Barré syndrome which is neurological disorders with paralysis (61-63). *Campylobacter* infections have been reported at any time of year and most cases are sporadic. In developing countries, the infections occur more frequency in children with an age < 5 years old with isolation rates of up to 18% (64).

Campylobacter are commensally in alimentary tracts of various warm-blooded animals, especially birds and commonly contaminate the environment, including surface water. Sources of contamination can be in the production chain of food, raw materials, dry stainless steel surfaces, instruments, or humans with transmission rate of 20% to 100% (65). Pets such as dogs, cats and birds may a reservoir of campylobacters (66). Most *Campylobacter* infections are related with a consumption of undercooked broiler meat which is regarded as a significant source of human infections. Additionally, the infections can be transmitted through contaminated untreated water, raw milk and other food products. Reducing counts of *Campylobacter jejuni* on chicken carcasses, in milk or food can significantly decrease an incidence of human campylobacteriosis (67). Prevalence of *Campylobacter* in poultry is high, ranging from 40 to 100% (68). Poultry are recognized as a major reservoir of *C. jejuni* because the high body temperature of birds enhances the bacterial colonization. Poultry colonized with campylobacters usually have no symptoms of illness and long term carriage is commonly occurred. Poultry carcasses can be contaminated by intestinal contents that spread to the carcasses during slaughter or by cross contamination during processing in the plant (69). *Campylobacter* found in water and soil, should be due to fecal contamination by wild or domestic animals that

water and soil, should be due to fecal contamination by wild or domestic animals that are natural reservoir of the microorganisms. The organisms could survive in stream water for up to four weeks and in soil for 20 days under a cold temperature of 4-6°C. Therefore contaminated environments could be potential sources of *Campylobacter* transmission (70, 71). In Lancashire, England, livestock including chicken and cattle attributed to the majority (97%) of sporadic *Campylobacter* infections while other animals (e.g. sheep, pig, wild bird, and wild rabbit) and environmental water sources were less likely (3%) than chicken and cattle. The study indicated that the primary transmission route is through the food chain; therefore on-farm biosecurity and implementation of risk assessment programs essentially reduced food-borne transmission (72). In processing plants, application of acid sprays, irradiation methods, chlorine and hot water rinses, or post-chilling methods are generally practiced to reduce and control *Campylobacter*. However, contamination of chicken products still occurred based on a recovery of *Campylobacter* from raw meats in retail markets (73). More effective pre-harvest and post-harvest control methods are required to eliminate *Campylobacter* on the farms and during food processing for ensuring the safety of food products (74).

2.4 *Campylobacter* pathogenesis

The complete genome sequence of *C. jejuni* NCTC 11168 attributes a progression of studying molecular mechanisms in *Campylobacter* pathogenesis (75). To cause a disease, *C. jejuni* has to survive inside host environmental stresses and in external physiological stresses, such as hostile temperatures and pH, oxidative stress, host immunity and nutrient constraint (76, 77). After a consumption of contaminated foods or water, campylobacters that can survive from stomach acid enter host intestine and colonize the epithelium of the distal ileum and colons (78). The organisms penetrate a mucus layer of intestinal cells via their cork-screw motility with polar flagella (79). Several virulence factors related to *C. jejuni* pathogenesis include motility, chemotaxis, iron acquisition, adhesion and invasion, quorum sensing, superoxide stress defense, thermal-stress response, toxin production and an ability to avoid the host immune system (80, 81). From genome sequence, *C. jejuni* contains

uncommon constituent of bacterial surface structures and sialic acid residues are also found on human gangliosides. Therefore, this structure may be involved in evasion of host immunity through molecular mimicry and in post-infection autoimmune diseases, Guillain–Barŕe syndrome (82). Chemotaxis enhances *Campylobacter* to colonize on mucosal epithelium of intestine. This mechanism detects chemical gradients via a complex signal transduction pathway causing flagella rotation and cell movement towards or away from the gradient (83). *C. jejuni* has positive chemotactic response to salts of organic acids (e.g. pyruvate, succinate and fumarate), bile extract, mucin and its components, L-serine and L- fucose, while bile acids have chemorepellant effects (84). Pathogenic strains of *C. jejuni* produce enterotoxins and cytotoxins (85). Enterotoxins are likely responsible for watery diarrhea via an increasing in intracellular cAMP after the binding of toxins to host cell receptors. The genome of *C. jejuni* NCTC11168 contains genes encoding the cytolethal distending toxin (cdtA-C). CDT isolated from *C. jejuni* affected on various cell lines including CHO, Vero, HeLa and Hep-2 cells (86). Cytotoxins exert their activity in various ways such as by inhibiting protein synthesis and actin filament formation, or by forming pores in the membranes of target cells resulting in cell death by lysis (85).

2.5 Detection methods

Detection of *C. jejuni* or thermophilic *Campylobacter* can be accomplished by various methods depending on types and sources of samples. Detection of *Campylobacter* can also be accomplished depending on a goal of detection, preventive detection by detecting in food chain or in a process of food production, diagnosis detection by detecting in fecal specimens of patient during an onset of the disease, and prognostic detection for follow-up the patient's symptom or patient's immune status. Since the characteristics of the diseases caused by *C. jejuni* show symptoms of diarrhea, gastroenteritis and abdominal pain that can be found in other bacterial gastroenteritis. A rapid diagnosis for patient treatment is significantly required to choose an appropriate treatment for immediately compensation of unwanted signs and symptoms and for in-patients. Currently, there are many rapid detection methods and

rapid test kits developed in various formats which are commercially available worldwide.

Conventional cultural methods for *Campylobacter* detection form food products usually include sample preparation, preenrichment and enrichment steps, isolation on selective media, and identification using biochemical tests (12). Many formula of enrichment media have been developed and using different antibiotics. For example, Preston broth contains nutrient broth with additives including FBP (ferous sulfate, sodium metabisulfite and pyruvic acid) and lysed horse blood, and antibiotics, namely trimethroprime, polymyxin b, rifamipicin and cycloheximide (87). Preston medium was useful for detecting low number of campylobacters in samples. Bolton broth composes of hemin, lysed horse blood, cefoperazone, vancomycin, trimethoprim lactate and amphotericin B (12). Bolton broth is a recommended enrichment broth according to ISO 10272-1: 2006. Selective plating media for isolation of *Campylobacter* are Skirrow agar (88), Campy-BAP agar (89), Preston agar (90), modified charcoal cefoperazone deoxycholate agae (91), Butzler agar (92), Karmali agar (93), and semi-solid agar (94). These media have been subjected to comparative studies with clinical and food samples. Generally, an enrichment step of traditional methods requires 1-2 days of incubation, then 2 days for incubation of selective agar plates and next 1-2 days for identification by biochemical tests.

Antibody-based methods for *Campylobacter* have been developed by using monoclonal or polyclonal antibodies incorperated in the detection systems. A simple and rapid method to detect *Campylobacter* spp. in chicken samples called NH Immunochromato-*Campylobacter* (NH IC Campy, Nippon Meat Packers, Ibaraki, Japan) was developed. This method consisted of a combination of a two-step enrichment method to detect *Campylobacter* antigen in an enrichment culture within 15 min. The method did not require blood or a particular system of generating a microaerobic atmosphere during the enrichment step, in contrast to the standard method of enriching *Campylobacter* spp. in chicken samples. The sensitivity for detection of non- and freeze-stressed *Campylobacter* spp. in spiked chicken was ranged from 5.5 to 1.3×10^1 cfu/ 25g and 9.2×10^1 to 1.5×10^2 cfu/25g, respectively (95). Antigen-antibody based assay was also developed commercially named Singlepath® *Campylobacter* test which involved 24 h of enrichment in Bolton broth with PCR-

based-identification. The test was positive with *C. jejuni* only and the detection limit was 10^3 cfu/mL. *Campylobacter coli* was undetectable at the the concentration of 10^5 cfu/mL while PCR provided the same detection limit of 10^2 cfu/mL for both of *C. jejuni* and *C. coli*. (96).

Nucleic acid-based techniques have been widely developed for rapid detection of *Campylobacter* such as PCR, real-time PCR and probe hybridization techniques. Traditionally, fluorescence *in situ* hybridization (FISH) has been developed with labeled DNA oligonucleotide probes and the use of a high affinity peptide nucleic acid (PNA) oligonucleotide sequence for detecting thermotolerant *Campylobacter*. PNA overcame the problem of the lack of affinity due to the location of the binding site and the variation of the target sequence within species. The PNA probe did not hybridize *Campylobacter* in coccoid form, indicating that the probe may be used to assess the physiological status of targeted cells samples (97). A real-time PCR assay for quantitative detection of *C. jejuni* in naturally contaminated poultry , milk and environmental samples without an enrichment step was developed for the detection of *C. jejuni*. The whole assay could be completed in 60 min with a detection limit of approximately 1 cfu. The real-time PCR assay was a specific, sensitive and rapid method for quantitative detection of *C. jejuni* (98). A multiplex-seminested polymerase chain reaction assay (m-sn PCR) was developed which enables the simultaneous identification of 4 major foodborne pathogens, i.e., *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter jejuni*, *Campylobacter coli* and enterohemorrhagic (eae gene positive) *Escherichia coli.* The first step of the multiplex PCR alone was capable to detect 10^3 to 10^4 cfu/mL for each species and using the second step seminested PCR signal amplification could lower the species-specific detection limits to 1-10 cfu/mL for *E. coli*, 1 cfu/mL for *L. monocytogenes*, 1-10 cfu/mL for *C. jejuni, C. coli* and 1 cfu/mL for *Salmonella* spp. (99). DNA microarray for detection of *Campylobacter* spp. was developed and applied to directly detect *Campylobacter* spp. from chicken feces. The results obtained with DNA microarrays compared to those obtained by conventional culture and gel electrophoresis revealed that 60% of the samples were positive for *C. jejuni* and *C. coli* by conventional culture and 95% of the samples were positive by PCR and capillary electrophoresis whereas by DNA microarrays all samples were positive for *Campylobacter* spp. (100).

2.6 Applications of magnetic nanoparticle

Magnetic nanoparticles are a class of nanoparticle which can be manipulated using magnetic field. Such particles commonly consist of magnetic elements such as iron, nickel and cobalt and their chemical compounds. Nanoparticles are smaller than 1 micrometer in diameter (typically 5 - 500 nanometers) while the larger microbeads are 0.5-500 micrometers in diameter (101). The magnetic nanoparticles are recently the focus of many researches because they possess attractive properties which have potentially used in catalysis, biomedicine, magnetic resonance imaging, magnetic particle imaging, data storage and environmental remediation. The existence of the following important properties is attractive for use in many applications (25). Firstly, magnetic nano- and microparticles can be selectively in separation from the complex samples by using an external magnetic field (e.g. using an appropriate magnetic separator, permanent magnet, or electromagnet). That process is important for applications because absolute majority of biological materials have diamagnetic properties, which enable efficient selective separation of magnetic and magnetically modified materials. Secondly, magnetic nano- or microparticles can be targeted to the desired place and collected using an external magnetic field. These properties can be used for example in the course of magnetic drug targeting. Thirdly, magnetic nano- or microparticles can generate heat when subjected to an alternating magnetic field which this phenomenon is employed especially during magnetic fluid hyperthermia. Fourthly, magnetic iron oxides nanoparticles generate a negative T2 contrast during magnetic resonance imaging thus serving as efficient contrast agents. Fifthly, magnetic nano- and microparticles can be used for magnetic modification of diamagnetic biological materials (e.g. cells) and magnetic labeling of biologically active compounds (e.g. antibodies and nucleotide probes).

Applications of magnetic nanoparticles for detection of biological molecules have been intensively reviewed (25). A combination of magnetic capture hybridization with PCR assay has been applied to detect many paghogenic bacteria such as *Listeria monocytogenes* in milk (102), *Salmonella* species in water (103), verotoxigenic *Escherichia coli* in ground beef (35) and *Mycobacterium avium* in feces (104). These methods demonstrated an improvement of detection limits of the PCR assay that have been used with complex sample matrices. Magnet particles conjugated

with specific antibodies for capturing *Campylobacter* cells prior to PCR assay were developed for detection in various samples, including milk, chicken products (27, 105-107) and chicken feces (28).

2.7 DNA dielectrophoresis

Dielectrophoresis (DEP) is the induced motion of an polarizable particles in non-uniform electric fields. DEP is widely applied to manipulate and separate various biological microparticles, such as cells, nucleic acids, proteins, carbohydrates and lipids. DEP is non-destructive and label-free method; therefore, after manipulation by DEP, the particle properties remain unchanged and appropriate for further analysis. Dielectrophoresis is derived from two words: *dielectro* meaning the dependence of the motion on the dielectric properties of the body; and *phoresis* meaning motion. Particle scales between nanometres and milimetres can be applied by using DEP. DEP can be used for manipulating nanoscale objects and trapping the particles in a certain position. For a micrometer or larger scale, DEP is commonly combined in a separation technique for bioparticles such as cells, viruses and proteins (108, 109). The electricity applied on DEP can be alternate current (AC), direct current (DC), or combined AC/DC which have different advantages of applications. For AC-DEP, electrode array is used to generate a spatially non-uniform electric field and particles are separated by adjusting the medium property and frequency of the electicity. Additionally, a driven flow within a chamber device is forced by an external pumping and the dielectrophoretic force exponentially decreases in proportional to a distance away from the electrode surfaces (38). Advantages of AC-DEP are that: (i) electrophoretic and electroosmotic effects are not presented in the system, (ii) low electric potentials are needed to generate equivalent electric effects, and (iii) low conductive medium is required for separation of bioparticles. For DC-DEP or insulator-based DEP, an insulator is used for generating the spatially inhomogeneous electric field. The DC electricity can create electroosmotic force, so an external pumping is not required for the system. Advantages of using insulating posts or constrictions are that: (i) electrodeless DEP, (ii) less complex fabrication process, (iii) less bubbles inside separation channels, and (iv) no metal surface deterioration during

sample analysis (39). In contrast to AC dielectrophoresis system, the DC DEP devices requires larger electric potentials and higher conductive medium in separation the particles. Recently, many researches proposed novel designed micro- or nanofluidic devices using dielectrophoretic forces for manipulation of particular biological particles. Advantages of the microdevices are small volume requirement, low power utilization, short operation time, low cost, portability, and potential for integration with other devices (110).

The principle of dielectrophoresis is demonstrated in Figure 2.1. Generally, surface charges of polarizable objects are induced in an electric field which the positive and negative charges are gathered on the opposite sides of the object. This phenomenon has the Coulombic interaction between the surface charges and the electric field. In a uniform electric field (Figure 2.1A), the particle is immobilized because of no net force. However, in a non-uniform electric field (Figure 2.1B), electrostatic forces on the accumulated charges are not equal so the net force drives the particle toward higher or lower electric field area depending on the particle properties and a surrounding medium (40). DEP contains two types, positive and negative. In positive DEP, a particle has higher polarizability than the surrounding medium so the net force pushes the particle towards the higher electric field region. In negative DEP, a suspended particle has smaller polarizability than the medium so the particle is pushed toward the lower electric field. Therefore, the resulting of DEP migration of the polarizable particles (both charged and neutral particles) is either moved towards the high electric field region or moved towards the low electric field area. Various parameters have been recognized to affect the dielectrophoretic forces acting on a particle motion, including charge of the particle, platform of the system, physiology of the particle, and dielectric constant of the medium and particle. To achieve a high yield of particle manipulation, the frequency and magnitude of the electric field and the conductivity of the suspending medium have to be intensively optimized. The conductivity of the surrounding medium should be relatively low for preventing the heat generation and for providing a high polarization of the particle over the polarization of the suspending medium. To achieve high particle trapping, high frequencies, in MHz, should be applied for preventing electrophoretic and electroosmotic effects. However, the higher frequencies were applied, the higher

voltages were used which causes a reduction of the general polarizability. During the experiment, the trapping DEP potential should be adjusted enough to overcome the Brownian motion of the particles in the suspending medium (40).

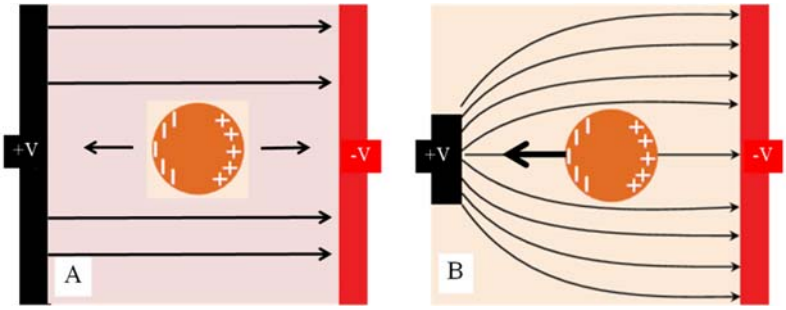


Figure 2.1 Schematic picture of dielectrophoresis.

AC-DEP is a classical DEP technique that uses metal electrodes to generate spatially non-uniform electric fields. Various applications of AC-DEP have been reported such as the separation of colloidal particles (111, 112), biological cells e.g. pancreatic islets, cancer cells lines, and blood cells (113-116). Interdigitated castellated electrode arrays have been effectively applied for the separation of bacteria, yeast and plant cells (117). After various cell populations were introduced into the electrode array and different frequencies of electric field were continuously applied, each cell types could be separated by positive and negative dielectrophoresis. The particles of positive DEP are accumulated to regions of the highest electric field which usually are electrode edges or gaps between adjacent electrodes. While the particles with negative DEP are trapped in areas of less electric field. The castellated electrode design was successfully applied to separation of live, dead and dormant *Mycobacterium smegmatis* cells, based on their different morphological and physiological properties at different states. The dead cells showed the highest conductivity while the live cells showed the lowest conductivity. At 20 V peak-to-peak (pp), 120 kHz frequency and 2 µS/cm, the active cells were moved toward the electrode tip because of positive DEP whereas the dorman cells were attached on the top of the electrode surface by negative DEP. At 20 V_{pp}, 80 kHz frequency and 900 µS/cm, the dead and dormant cells were separated. The dormant cells were aggregated

between the electrode regions by negative DEP while the dead cells stayed in the chamber because of the positive DEP (118). Using interdigitated electrode design, the mouse melanoma cells with a diameter of 20 μm could be separated by the their significant different melanin content which is useful for determining metastatic levels of cancer. At 5 V_{pp} and a frequency of 300 kHz, one clone experienced negative DEP while another clone experienced positive DEP. Under the applied electric frequencies at 200 and 400 kHz, both clones exhibited negative DEP and positive DEP, respectively (119).

Application of dielectrophosis for manipulation of deoxyribonucleic acid (DNA) including DNA trapping, stretching and separation has been widely reported, however, the detail mechanism of DNA polarization remains unclear (120). A simple explanation for a long DNA in an electrolyte solution is that the counterion cloud surrounding a coiled DNA conformation shifts in an electric field leading to DNA polarization. The polarizability depends on DNA conformation (linear or supercoiled) and size of the DNA molecules. Generally, the polarizability increases with DNA length, so the small size of DNA which presumably smaller polarizability should require high electric field gradients in order to achieve DEP migration (37, 121). A wide range of DNA from 20 bases to several kilobases can be manipulated by DEP depending on the size of the electrodes used. Methods for measuring DEP movement are using fluorescence microscopy and impedance spectroscopy. Fluorescent microscopy is considered as a standard method for visualizing and measuring DNA dielectrophoresis (122). Many proper fluorescent dye including DAPI, YOYO, TOTO, acridine orange and PicoGreen are used to interaction on dsDNA. However, the technique requires sample preparation which is laborious and the fluorescent dyes may act as artifacts with the interesting DNA molecules and with the electrical field. Additionally, the fluorescnece is faded with time because of photobleaching of the dye and the DNA strand may be broken up because of photoscission (123). Impedance spectroscopy or capacitance measurement is a label-free method which uses the same electrodes for manipulation and for sensing of the DNA. The presence of DNA molecules can change the capacitance between the measuring electrodes. An creative arrangement of the electrodes for DEP was developed for measuring of DNA movement and DNA quantification (124, 125). DNA dielectrophoresis and impedance

measurement can be intermittently analyzed through a component analyzer which serves for both DEP excitation and measurement. In this combination system, the redistribution of DNA molecules after dielectrophoretic accumulation occured on a much longer time period than switching between DEP and sensing mode (120).

CHAPTER III
MAGNETO-PCR-ENZYME LINKED GENE ASSAY FOR DETECTION OF THERMOPHILIC *CAMPYLOBACTER* SPECIES

3.1 Abstract

Thermophilic *Campylobacter* including *C. jejuni*, *C. coli* and *C. lari* are important food-borne pathogens causing an acute gastroenteritis worldwide which infectious cases mostly related to a consumption of undercooked poultry or handling with raw poultry products. This study developed a magnetic-based PCR assay for rapid detection of thermophilic *Campylobacter* by using the biotinylated-reverse primer bound to streptavidin-horseradish peroxidase and its substrate for detection of the PCR products. The forward primer conjugated to the magnetic nanoparticles facilitated separation and concentration of the target bacterial DNA leading to high sensitivity (1 pg per PCR reaction) and specificity. When the proposed technique was preliminary applied to examine the naturally contaminated chicken skin, all positive results were in accordance with the results from standard cultural method and conventional PCR. The magneto-PCR-enzyme-linked gene assay is practical and possibly useful for detection of thermophilic *Campylobacter* from samples containing complex matrices and PCR-interfering substances.

3.2 Introduction

Thermophilic *Campylobacter* species, mainly *C. jejuni*, are major causative agents of bacterial gastroenteritis with inflammation, abdominal pain, fever and diarrhea (1, 2). Post-infections of *C. jejuni* are associated with low incidence rates of Guillain-Barré syndrome and reactive arthritis (61-63). In contrast to developed countries, high prevalence of *Campylobacter* infection in children in developing countries was occurred with 5.5%-18% isolation rates from diarrheic specimens of children less than 5 years old (64). *Campylobacter* isolation rate in Thailand was about

13% (8, 9) with a rate of 18.8% at the age of <12 months; 12.3% at the age of 12-23 months; and 10.3% at the age of 24-59 months (10). Resistance of *Campylobacter* to fluoroquinolones and macrolides has increasing throughout the world in the last decade. The relationship between drug utilization in food animals and resistant *Campylobacter* isolated from human has been intensively reviewed (126, 127). Campylobacteriosis has been related with a consumption of raw milk, surface water and poultry products (3). *Campylobacter* spp. colonizes in intestinal tracts of domestic and wild animals (128) and chicken is considered as a vital natural source of human infections. High numbers of *C. jejuni* colonizes in intestines, especially in ceca, of chicken without causing any symptoms (2). *Campylobacter* species could be isolated from retail poultry meat with various ratios of *C. jejuni* to *C. coli* among the countries. Approximately 71%, 58% and 69% of retail poultry meat were contaminated with *Campylobacter* spp. in the USA, Japan and UK, respectively (129). In Thailand, thermophilic *Campylobacter* were isolated in 12-65% of chicken samples with a reported ratio of *C. jejuni*:*C.coli*:other thermophilic *Campylobacter* in 24:65:11 (4-7).

Conventional cultural techniques for *Campylobacter* detection are time-consuming and labor-intensive (12). However, they are recognized as a gold standard method. The culture method allows phenotypic and genotypic analyses of the isolates. A rapid detection method is required for investigating of *Campylobacter* infections and detecting as a screening of negative samples during chicken processing. Nucleic acid-based methods are needed for identification of a viable but non-culturable state of *Campylobacter* cells which could not be detected by the culture technique (13). PCR-based assays for *Campylobacter* have been developed because of high sensitivity and specificity (14, 16-19, 130). After gel electrophoresis, PCR products are generally detected by staining with ethidium bromide, known as a carcinogenic agent, and visualizing the amplicons in agarose gel. While real-time PCR requires an expensive analyzer which is not commonly available at the field sites or at local microbiological laboratories. Immunological detection methods are practical to operate and a positive result is easily observed by a color development from a chemical reaction. Enzyme-linked immunosorbent assay for *Campylobacter* detection from stool specimens were developed but the assays still had high cross-reactivity and low sensitivity (131, 132). A combination of an immunoassay and PCR, called immuno-PCR technology, was

proposed with a high sensitivity and a broad variety of applications (133), including an incorporation with an advantage of magnet nanoparticles in a capture phase (101). Magnetic nanoparticles have various applications for detection of biological molecules (25). The magnetic beads conjugated with antibodies or oligonucleotides have been developed for selective removal of target molecules from complex samples using an external magnetic field. Magnet particles conjugated with specific antibodies used to capture *Campylobacter* cells prior to PCR were developed for detection from milk, chicken products (27, 105-107) and chicken feces (28). Protocols for immobilizing of primers on magnetic nanoparticles were successful developed with various detection systems (31-34). Here, we applied an advantage of the magnetic-based PCR with enzyme-linked gene technique for *Campylobacter* detection.

More than 16 *Campylobacter* species have been identified but thermophilic species are most clinically recognized infection (134). Most cases of campylobacteriosis caused by *C. jejuni* which significant higher than *C. coli* and *C. lari*. While *C. upsaliensis* are less common cause of human foodborne infections (3). This study designed to detect *C. jejuni*, *C. coli* and *C. lari* by selecting PCR primers targeting 16S rRNA that had been validated previously (135-137). In this study, an enzyme-substrate detection system coupled with magnetic-based PCR was set up for thermophilic *Campylobacter* detection. The specific forward primers bound to the magnetic nanoparticles and the reverse primers labeled with biotin were used in the PCR assay. After PCR amplification, the amplicons attached to the nanoparticles were collected and detected the generating colored products by using streptavidin-horseradish peroxidase and its substrate. The schematic of the detection of *Campylobacter* DNA by the MNP-PCR-enzyme linked gene technique is summarized in Figure 3.1.

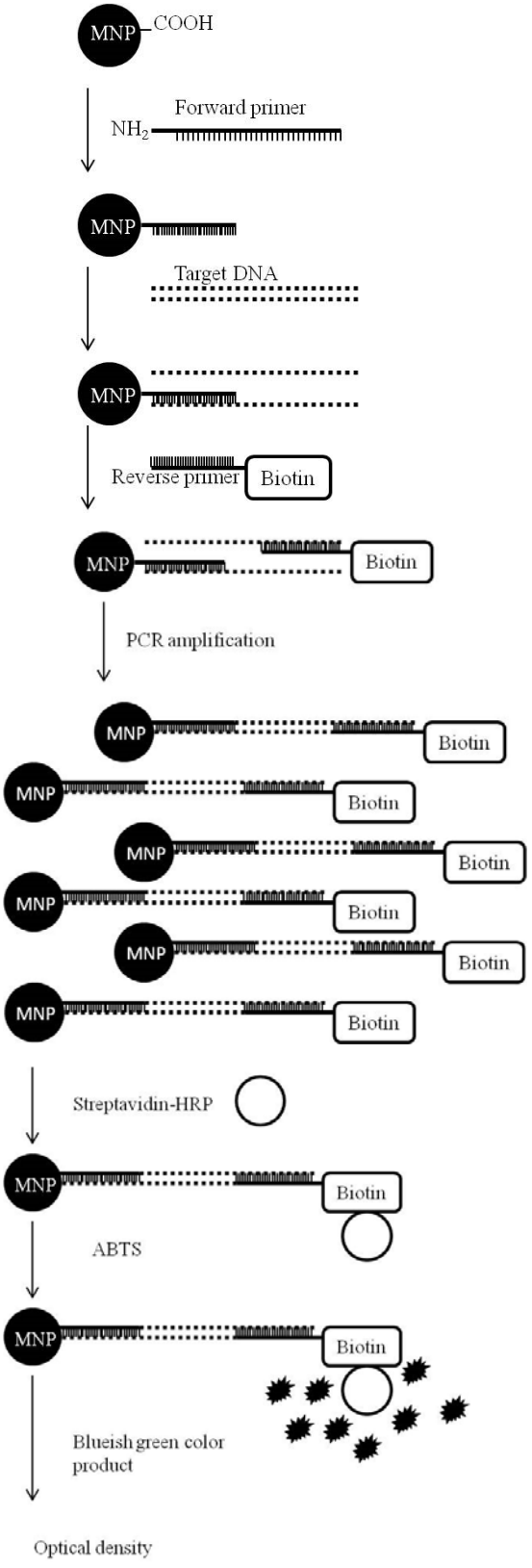


Figure 3.1 Schematic of the detection of *Campylobacter* DNA by MNP-PCR-Enzyme linked gene technique.

3.3 Materials and Methods

3.3.1 Bacteria strains and culture condition

C. jejuni ATCC 33291, *C. jejuni* ATCC 33560, *C. coli* MUMT18630, *C. lari* ATCC 43675, *C. upsaliensis* DMST19055, *C. fetus* ATCC 27374 from stock cultures were subcultured on Charcoal deoxycholate agar (Oxoid LTD., Hampshire, England) in a microaerobic atmosphere at 37°C or 42°C for 18-24 h. Non-*Campylobacter* bacteria including *Acinetobacter baumannii, Aeromonas hydrophila, Bacillus cereus, Burkholderia cephacia, Corynebacterium xerosis, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Pseudomonas alcaligenes, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella paratyphi A, Shigella sonnei, Streptococcus agalactia, Vibrio parahaemolyticus* and *Yersinia enterocoliticus* were cultured overnight on Tryptic soy agar (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C under aerobic condition.

3.3.2 Bacterial DNA extraction

Bacterial DNA was extracted from isolated colonies by using Trizol® (Invitrogen, Ontario, Canada) following the manufacturer's manual instruction. Briefly, bacterial cells were suspended in 1 mL of Trizol® reagent and incubated for 5 min. Chloroform (1 mL) was added and the mixture was centrifuged at 10000x *g* for 15 min at 4°C. The colorless upper aqueous phase was collected and added with an equal volume of absolute ethanol to precipitate DNA. The mixture was centrifuged at 2000x *g* for 5 min at 4°C and DNA pellet was washed twice with 0.1 M sodium citrate in 10% ethanol. The DNA pellet was then suspended in 75% ethanol and stored at room temperature for 10-20 min. After centrifugation, the DNA was dried at ambient atmosphere and dissolved in sterile distilled water.

3.3.3 Immobilization of primers on magnetic nanoparticles

The carboxylated magnetic nanoparticles with a diameter of 250 nm (Chemicell, Berlin, Germany) were immobilized with the NH_2-modified forward primers according to the covalent coupling procedure by carbodiimide method

suggested by the manufacturer. Briefly, MNPs were washed twice with 500 µL of 25 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 6.0, and then 1 mg of the MNPs was mixed with 5.4 nmol of designed forward primer (Table 1). After incubating under gentle shaking (900 rpm) at room temperature for 30 min, 10 µL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution (10 mg EDC in 0.25 mL MES buffer) and MES buffer were subsequently added to make a final volume of 100 µL. After incubating at 4°C for 2 h with gentle shaking, the immobilized MNPs were separated by magnetic separator and incubated with 50 mM Tris buffer (pH 7.4) under gentle shaking at room temperature for 10 min to quench the unreacted carboxyl groups (138). The primer-immobilized MNPs were washed twice and then suspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) before storage at 4°C.

3.3.4 Immobilization efficiency testing

An efficiency of the coupling reaction to produce the primer-immobilized MNPs was indirectly tested by hybridization with a complementary primer tagged with biotin (Table 1). Thirty microliters of 0.5 µM primer-coupled MNP was gently washed twice with 100 µL of distilled water and magnet was used to hold the MNPs during separation. Then, 3' biotin labeled complementary primer with a final concentration of 0.5 µM in 13x SSC/0.05% BSA was added and the suspension was incubated for 30 min at room temperature. The MNPs was washed twice with 100 µL of distilled water and incubated with 50 µL of horseradish peroxidase-conjugated streptavidin at room temperature for 60 min. After washing with distilled water, 50 µL of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) containing H_2O_2 (50%) (Kirkepaard & Perry Laboratories, Gaithersburg, MD, USA) was added and then, incubated for 30 min at room temperature. Enzyme activity was measured by the Nanodrop 2000 (Wilmington, DE, USA) at 405 nm and expressed as an optical density (OD) value. Distilled water instead of biotin-labeled complementary primer was used as negative control. Another negative control was primer-uncoupled MNPs instead of primer-coupled MNPs.

3.3.5 MNP-based PCR

Two primers were designed for PCR amplification of *C. jejuni* 16S rRNA (GenBank accession no.Y19244) which was specific for detection of thermotolerant campylobacters (135). The NH_2-modified forward primer was immobilized on MNPs and the backward primer was labeled with biotin as shown in Table 3.1. PCR was carried out in a mixture (30 µL) containing 1X *Taq* buffer, 0.5 µg/µL BSA, 3.0 mM $MgCl_2$, 0.3 mM of each deoxynucleotide triphosphates (dNTP), 1 U of *Taq* DNA polymerase (Fermentas, Maryland, NY, USA), 0.5 µM of unbound or MNP bound primers (3 µg MNP content) and 1 µL of DNA sample. The amplification profile was as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 1 min; final extension at 72°C for 5 min. The MNPs-PCR products were determined by enzyme-substrate system or by fluorescence intensity. DNA of *C. jejuni* ATCC 33291 and distilled water were used as positive and negative controls, respectively, in MNP-based PCR assay.

Table 3.1 Primers used (135)

Primer	**Sequence (5' to 3')**	**Product size (base pairs)**
Forward	NH_2-C_{12}-CTGCTTAACACAAGTTGAGTAGG	
Backward	Biotin-TTCCTTAGGTACCGTCAGAA	287
C-Forward*	Biotin-GACGAATTGTGTTCAACTCATCC	

* Complementary forward primer

For enzyme-substrate system, the MNPs-PCR products were washed twice with distilled water and then incubated with 50 µL of horseradish peroxidase-conjugated streptavidin (SA-HRP) at room temperature for 60 min. The obtained SA-biotin complexes were washed twice with 100 µL of distilled water and simply separated by applying an external magnetic field. Then, 50 µL of ABTS containing H_2O_2 was sequentially added and the mixture was incubated for 30 min at room temperature. Enzyme activity was measured by the Nanodrop 2000 at 405 nm and

expressed as an OD value. For detection by fluorescence intensity, the MNPs-PCR products were washed twice with 100 µL of distilled water and then incubated with 50 µL of CF488A-labeled streptavidin (Biotium, Hayward, CA, USA) at room temperature for 60 min in the dark. The obtained SA-biotin complexes were washed twice with 100 µL of distilled water and finally resuspended with 50 µL of distilled water. The fluorescence intensity was measured at an excitation wavelength of 490 nm and emission wavelength of 515 nm.

3.3.6 Sensitivity and specificity determination of the MNP-based PCR

Serial ten-fold dilutions of *C. jejuni* ATCC 33291 DNA were prepared and 1 µL of each dilution was used as a DNA sample in the MNP-based PCR. The MNPs-PCR products were determined by both enzyme-substrate system and fluorescence intensity as mentioned above to evaluate their analytical sensitivity. For specificity determination, extracted DNA (1 ng) from 16 strains of non-*Campylobacter* bacteria was pooled into four groups (4 strains for each group) to use as DNA templates in the MNP-based PCR detected by the enzyme-substrate system. Conventional PCR reactions using forward and backward primers without labeling as shown in Table 1 were also used for amplification of the extracted DNA from all groups. The reaction of the conventional PCR was as follows: 95°C for 3 min; 40 cycles of denaturing at 95°C for 30 s, annealing at 51°C for 30 s, extending at 72°C for 1 min; and final extension at 72°C for 5 min. The 287 bp amplification product was expected from a positive control. PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

3.3.7 Detection in naturally contaminated chicken skin

The magneto-PCR-enzyme linked gene assay was compared with the conventional PCR and a cultural method for detection of campylobacters from raw chicken carcass samples. Chicken upper wings were purchased from different retailed markets and stored at 4°C for less than 3 h until analysis. Skin from chicken carcass was aseptically removed by random cutting with sterile scissors into small pieces. Cultural method for detection of *Campylobacter* spp. was as following. Ten grams of the skin samples were aseptically transferred into 90 mL of Bolton broth (Oxoid LTD.,

Hampshire, England) supplemented with antibiotics (cefoperazone, vancomycin, trimethoprim lactate and amphotericin B). The cultures were homogenized by shaking for 1 min and incubated in a microaerobic condition (at 5%O_2, 10% CO_2 and 85% N_2) at 37°C for 4 h, then at 41.5°C for 20 $\pm$ 2 h. After incubation, 2 loopfuls from enrichments were streaked on modified charcoal cefoperazone deoxycholate agar (Oxoid LTD., Hampshire, England) supplemented with cefoperazone and amphotericin B. The agar plates were incubated at 41.5°C for up to 48 h in a microaerobic condition and the plates were observed for typical colonies of *Campylobacter*. Characteristics for identification are small gram-negative curved bacilli and oxidase-positive bacteria. The enrichments were also collected for bacterial DNA extraction by alkaline boiling method. Briefly, enrichment broth (50 µL) was mixed with 50 µL of 50 mmol/L NaOH and boiled at 100°C for 10 min. Then, the mixture was mixed with 10 µL of 1 M Tris-HCl (pH 7.4) and centrifuged at 2,000x *g* for 1 min at room temperature. The supernatant was collected to use a DNA template for conventional PCR and the MNP-based PCR using enzyme-substrate system. Three separated experiments were performed for the MNP-based PCR using enzyme-substrate system for detection of campylobacters and the results were reported as mean $\pm$ standard deviation (SD).

3.4 Results

3.4.1 Primer immobilization

After primer-coupled MNPs were hybridized with a complementary primer labeled with biotin and detected by enzymatic reaction, the higher OD value was observed over those generated from negative controls (Figure 3.2). The specific primer designed with long spacer, $(CH_2)_{12}$, was successfully coupled on the surface of magnetic nanoparticles. Non-specific adsorption of complementary primers and streptavidin on MNPs was not significantly observed showing by very low OD value of the negative controls.

3.4.2 Sensitivity of MNP-based PCR

The analytical sensitivity was investigated by varying ten-fold dilution of *Campylobacter* DNA from 1ng/µL to 0.1pg/µL. The sensitivity of the fluorescent-based detection by measuring fluorescence intensity was observed at 0.1 ng (100 pg) of DNA (Figure 3.3A). The enzyme-substrate system showed the detection limit of 0.0001 ng (1pg) per reaction (Figure 3.3B) which was about 100-fold higher than that of fluorescent-based assay. Therefore, the MNP-based PCR using enzymatic detection system was used in the following experiments.

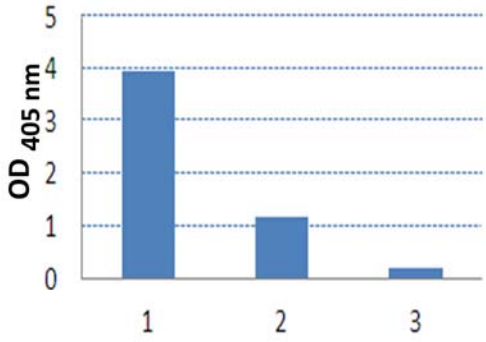
3.4.3 Specificity testing of MNP-based PCR assay

Extracted DNA from 16 non-*Campylobacter* bacteria was assayed by horseradish peroxidase-labeled streptavidin and ABTS® peroxidase substrate system in MNP-based PCR to determine the specificity as presented in Figure 3.4. OD values of the positive control (*C. jejuni* DNA) were significantly higher than those of non-*Campylobacter* bacteria (groups 1-4). Specificity testing by conventional PCR which analyzed by gel electrophoresis was also performed (Table 3.2). PCR product with a size of 287 base pairs was not observed from *Salmonella paratyphi* A, *Aeromonas hydrophila*, *Pseudomonas alcaligenes*, *P. aeruginosa*, *Enterobacter cloacae*, *Vibrio parahaemolyticus*, *Yesinia enterocoliticus*, *Listeria monocytogenes*, *Bacillus cereus*, *B. cephacia*, *Streptococcus* group D, *A. baumanii*, *C. upsaliensis* DMST19055 and *C. fetus* ATCC 27374, whereas, amplification of DNA extracted from *C. jejuni* ATCC 33291 and ATCC 33560, *C. coli* MUMT18630 and *C. lari* ATCC 43675 observed the target band (data not shown).

3.4.4 Detection in naturally contaminated chicken skin

The developing magneto-PCR-enzyme linked gene assay was preliminary applied to detect *Campylobacter* spp. from nine naturally contaminated skin samples that had positive results determinated by conventional cultural method and PCR assay. The results revealed that the assay was possibly used to detect of campylobacter contaminated in poultry carcasses with analytical sensitivity of 100% and specificity of 100% as shown in Table 3.2 and Table 3.3. The average of optical density from all nine positive samples were 0.60 $\pm$ 0.11 (mean $\pm$ SD) which significantly higher than

those of negative control (0.32 $\pm$ 0.03), Student's *t*-test with *p*-value < 0.0001, as shown in Figure 3.5.



Primer-coupled MNP	+	+	-
Primer-uncoupled MNP	-	-	+
Complementary primer	+	-	+
SA-HRP and ABTS	+	+	+

Figure 3.2 Immobilization efficiency test and reagents used.

The complementary primers tagged with biotin were used to hybridize with the forward primers immobilized on MNPs. The enzyme-substrate system (SA-HRP and ABTS) was used to evaluate an efficiency of the immobilization as represented by OD values. 1, primer-coupled MNP and complementary primer; 2, primer-coupled MNP only for checking non-specific binding of the detection system; 3, primer-uncoupled MNP and complementary primer for checking undesirable adsorption on nanoparticles' surface.

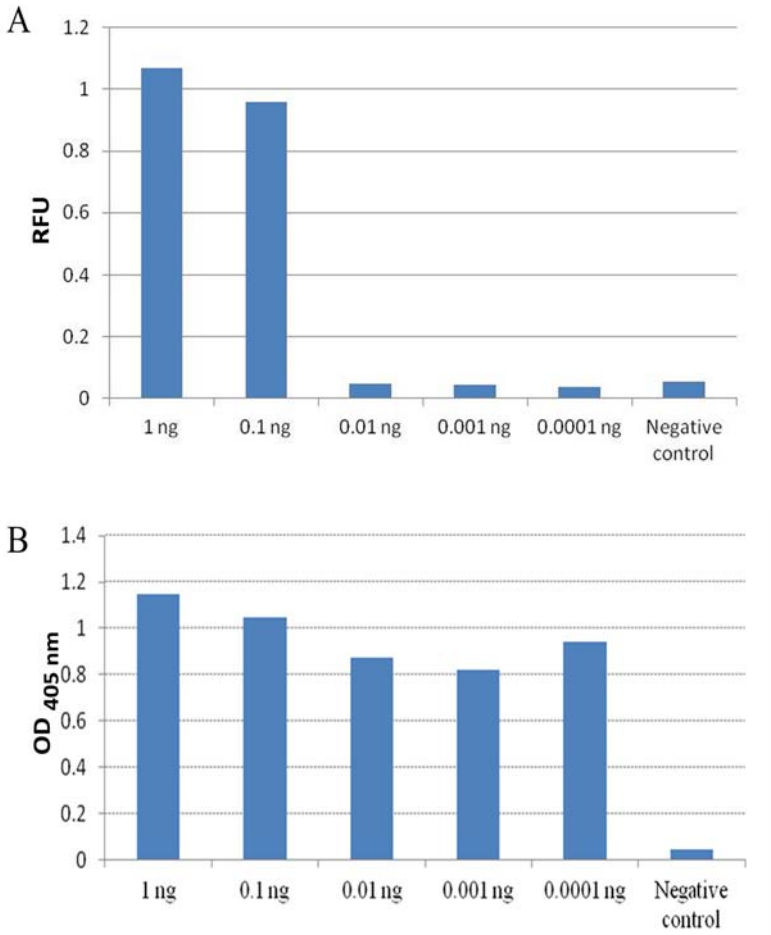


Figure 3.3 Sensitivity of MNP-based PCR.

Ten-fold serial dilutions of *C. jejuni* DNA ranging from 1 ng to 0.0001 ng were analyzed by MNP-based PCR and detected by CF488A-labeled streptavidin which presented as relative fluorescence unit (A), or detected by using streptavidin-horseradish peroxidase and ABTS which presented as an optical density (B).

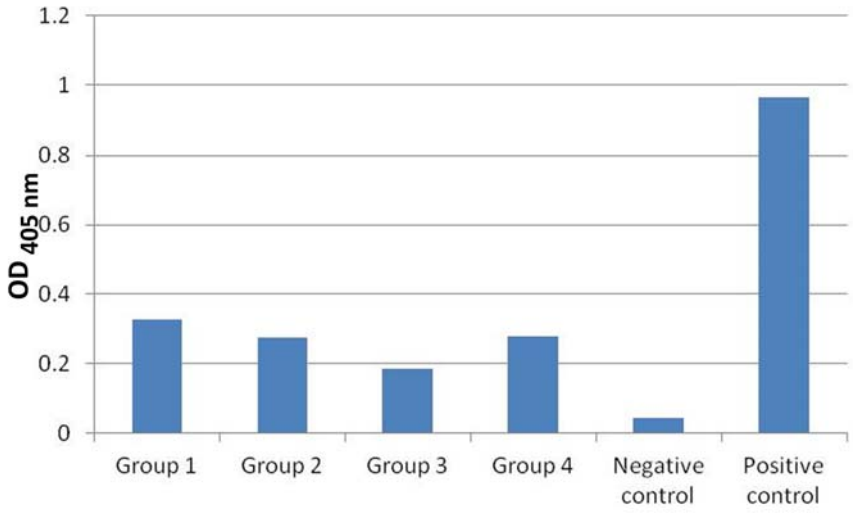


Figure 3.4 Optical density (OD) values of specificity determination.

Sixteen strains of non-*Campylobacter* DNA were pooled into 4 groups and analyzed by MNP-based PCR and the enzyme-substrate system. Distilled water and *Campylobacter* DNA were used as a negative control and positive control, respectively.

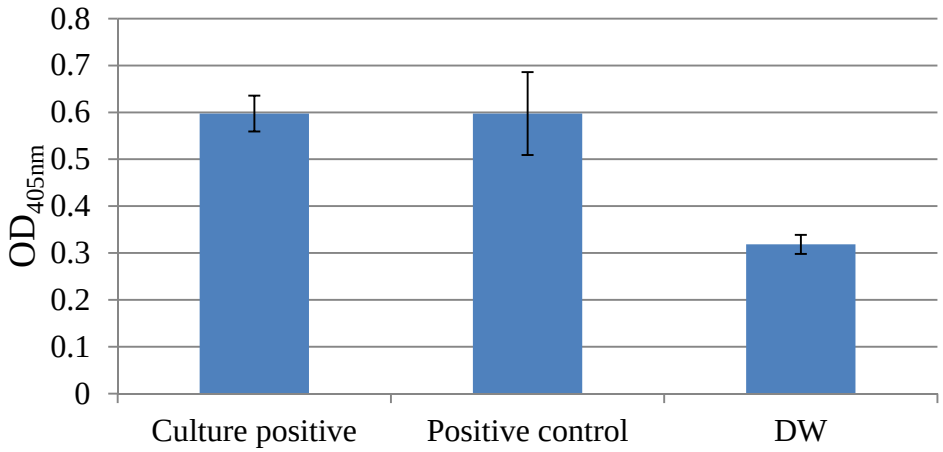


Figure 3.5 Average optical density (OD) values for *Campylobacter* detection in chicken skin samples. Natural contaminated skin samples (n=9) that culture-positive and PCR-positive were applied to the MNP-based PCR assay with detection by the enzyme-substrate system. *C. jejuni* DNA and distilled water (DW) were used as a positive control and negative control, respectively.

Table 3.2 Analytical performance of MNP-based PCR assay compared with conventional PCR

MNP-based PCR $OD_{405\ nm}$	**Conventional PCR**	
	Positive	Negative
Cutoff > 0.40; Positive	9*	0
Cutoff < 0.40; Negative	0	4**

* Chicken skin samples (n=9)

** Pure culture of mixed bacteria (groups 1-4)

Table 3.3 Analytical performance of MNP-based PCR assay compared with conventional cultural method

MNP-based PCR $OD_{405\ nm}$	**Culture method**	
	Positive	Negative
Cutoff > 0.40; Positive	9*	0
Cutoff < 0.40; Negative	0	3**

* Chicken skin samples (n=9)

** Negative controls (n=3)

3.5 Discussion

Conventional methods to detect *Campylobacter* spp. generally involve cultivation in enrichment media, isolation on selective media and identification by biochemical tests; however, these processes are cumbersome and require several days to diagnosis. Therefore, rapid, sensitive and specific techniques are challenging for ensuring efficient detection of the pathogens in food and clinical samples concerning for food safety and public health. Many molecular techniques, such as polymerase chain reaction (PCR), real-time PCR and loop-mediated isothermal (LAMP) were reported for identification of food-borne pathogens. New nanotechnology combined with traditional amplification assay has been extensively developed including immuno-PCR technology (133) and magnetic bead technology (139). In this research, specific forward primer was conjugated with magnet nanoparticles for *Campylobacter* DNA separation and amplification by PCR with detection by enzyme-substrate system.

In the PCR assay based on magnetic-capture hybridization, a spacer structure separates the $–NH_2$ linker from the oligonucleotide probe and the spacer length was reported to affect on hybridization efficiency of the assay. The longer spacer of 12 carbons was useful to avoid steric hindrance effects that may occur during capturing the target bacterial DNA (102). This study designed to use the long spacer, $(CH_2)_{12}$, linked with the forward primer which could be immobilized successfully on MNPs. Although, the primer set used in this study have been validated for their specificity (135), the specificity of our detection system was again confirmed with 16 strains of non-*Campylobacter* bacteria. The results revealed 100% specificity of the detection proposed. Similarly to this study, the magneto-PCR-engyme linked gene technique that was developed for detection of chronic myelogenous leukemia revealed the detection limit of 1 pg (140) and the sensitivity was also comparable to the real-time PCR technique (141, 142). The combination of MNPs and the enzyme-substrate system carried on an enhancement in the sensitivity of the developed system. To detect the amplification products in this study, the enzyme-substrate system demonstrated high sensitive (100 times) than the fluorescent-based system because of the catalytic nature of enzyme-mediated substrate conversion resulting in macroscopic

observation of the colored product. Moreover, this enzyme-substrate assay was more convenient and friendly than using the conventional gel electrophoresis.

Campylobacter contamination level on chicken products may be relatively low (143) so an enrichment step can facilitate the possibility of detection by increasing campylobacter cells and diluting inhibitory substances derived from food samples for the PCR-based assay. Enrichment media for *Campylobacter* contain inhibitory substances such as, immunoglobulin G (144), heme, lactoferrin (145, 146), bile, esculin (147, 148), charcoal and iron (149). An advantage of using magnet nanoparticles in the developed PCR assay should be benefit for applications to food samples containing interfering substances (150) and the high back-ground flora. High level of normal flora of chicken samples showed no negative effect on *Campylobacter* growth on Bolton broth and on PCR assay has been reported (135). A low volume of extracted DNA is generally used in a simple PCR detection, whereas, magnet nanoparticles immobilized with the primers assist to concentrate target DNA after DNA extraction leading to an increasing of *Campylobacter* detection.

The poultry skin is considered as a reservoir for *Campylobacter* with higher incidence of contamination than skinless meat (151). *Campylobacter* cells were mostly entrapped in skin crevices or feather follicles and could survive during storage at low temperatures (152, 153). In this research, the developed MNP-based PCR method was used for detection of natural contaminated *Campylobacter* in chicken skin and a simple alkaline boiling method was used for bacterial DNA extraction from overnight enrichment broth. All positive results form the magneto-PCR-enzyme linked gene method was in accordance with the standard cultural method according to ISO 10272-1: 2006. The development of magnetic nanobeads based PCR and detection by enzyme-substrate system for rapid and sensitive detection of thermophilic *Campylobacter* in this research is the first assay that was combined three standard technologies for investigation of poultry samples.

3.6 Conclusion

In this study, the sensitive PCR-based method using primer-conjugated magnetic nanobeads to separate and concentrate the target DNA and using enzyme-substrate system for rapid detection of thermophilic *Campylobacter* in pure culture and chicken skin was developed with the detection limit of 1 pg per PCR reaction. The assay demonstrated a good performance that was comparable to the standard cultural method and the conventional PCR for *Campylobacter* detection in chicken skin samples.

CHAPTER IV
DIELECTROPHORETIC IMPEDANCE MEASUREMENT METHOD FOR DETECTION OF PCR AND LAMP PRODUCTS

4.1 Abstract

Loop-mediated isothermal amplification (LAMP) is a technique for amplification of DNA without using an expensive thermal cycler machine. LAMP product is usually detected by turbidity measurement which occurs by magnesium pyrophosphate precipitation. Another detection method is to measure the intensity of dye that stains DNA molecules which requires spectrometry or device to measure after completing LAMP reaction. This study showed an application of dielectrophoretic impedance measurement (DEPIM) method for detecting LAMP and PCR products without any labeling of dyes or other molecules. PCR (189 bp) and LAMP products stained with SYTO9 were used for visualizing the DNA trapping on interdigitated microelectrodes by fluorescence microscopy. The DNA trapping was not observed after applying various frequencies and voltages. When measuring electrical conductance by DEPIM device, undiluted PCR product demonstrated high conductance of 6-7 μS/cm while control conductance was only 3-4 μS/cm. The conductance of undiluted LAMP product was about 40 μS/cm which much higher than that of the control (1.2 μS/cm). To lower a conductivity of the suspension medium either distilled water or ethanol were used to dilute the LAMP samples. At 1:10 dilution of LAMP product to distilled water, the conductance was 100-130 μS/cm after applying 150 kHz frequency and 3 V, while a control gave very low conductance of 7 μS/cm. At 1:3600 dilution with a conductivity of medium of about 20 μS/cm, the diluted LAMP product provided a conductance lower than a conductance of the control. At 1:1000 dilution of LAMP product to ethanol, the LAMP conductivity of 11 μS/cm was revealed while the control conductivity was only 0.8 μS/cm. This study showed a possibility of using DEPIM method for rapid detection of PCR and LAMP products by using ethanol or distilled water as a diluent.

4.2 Introduction

Loop-mediated isothermal amplification (LAMP) is a variant technique of PCR for increasing the copies number or amount of target DNA. The differences between LAMP, real-time PCR and conventional PCR methods are using isothermal temperature during amplification and using replacement ability of DNA polymerase for polymerizing a newly DNA strand which a newly DNA strand is polymerized continuously by polymerase reading of a template DNA strand. LAMP technique was employs strand displacement activity DNA polymerase and four specifically designed primers that recognize six different distinct sequences on target DNA. Two inner primers for initiation of LAMP and two outer primers for releasing a single-stranded DNA are served as a template in DNA synthesis as shown in Figure 4.1 (20). LAMP technique provides more sensitivity and practicality than a conventional PCR for detection of target DNA. In a comparison study, LAMP method could detect viral DNA better than real-time PCR with 80% increase in sensitivity (154). For detection of Japanese encephalitis virus, RT-LAMP assay gave more simple and less time-consuming than the conventional RT-PCR or real-time RT-PCR. The amplification could be completed in a single tube within 1 h under isothermal conditions and RT-LAMP assay can be a practical molecular diagnostic tool for JEV infection and various other virus infections (155, 156).

LAMP method is continuously applied in bacteriological or parasitological fields. The high sensitivity and specificity and less time-consuming are preferable when comparred to a conventional method or even to a gold standard method (22, 23). LAMP product can be detected via inspection of turbidity of reaction solution by naked eyes or an instrument. The by-product pyrophosphate ions that are produced during amplification can be used for monitoring the real-time amplification of DNA by combining with magnesium ions and a forming of a white precipitate of magnesium pyrophosphate. The difference of absorbance or precipitate amount at the beginning and the end points are useful for measuring and monitoring the dynamics process of LAMP reaction (157-159). Electrical based detection techniques could provided high sensitivity and more rapidity than does the optical density or naked eyes detection (160).

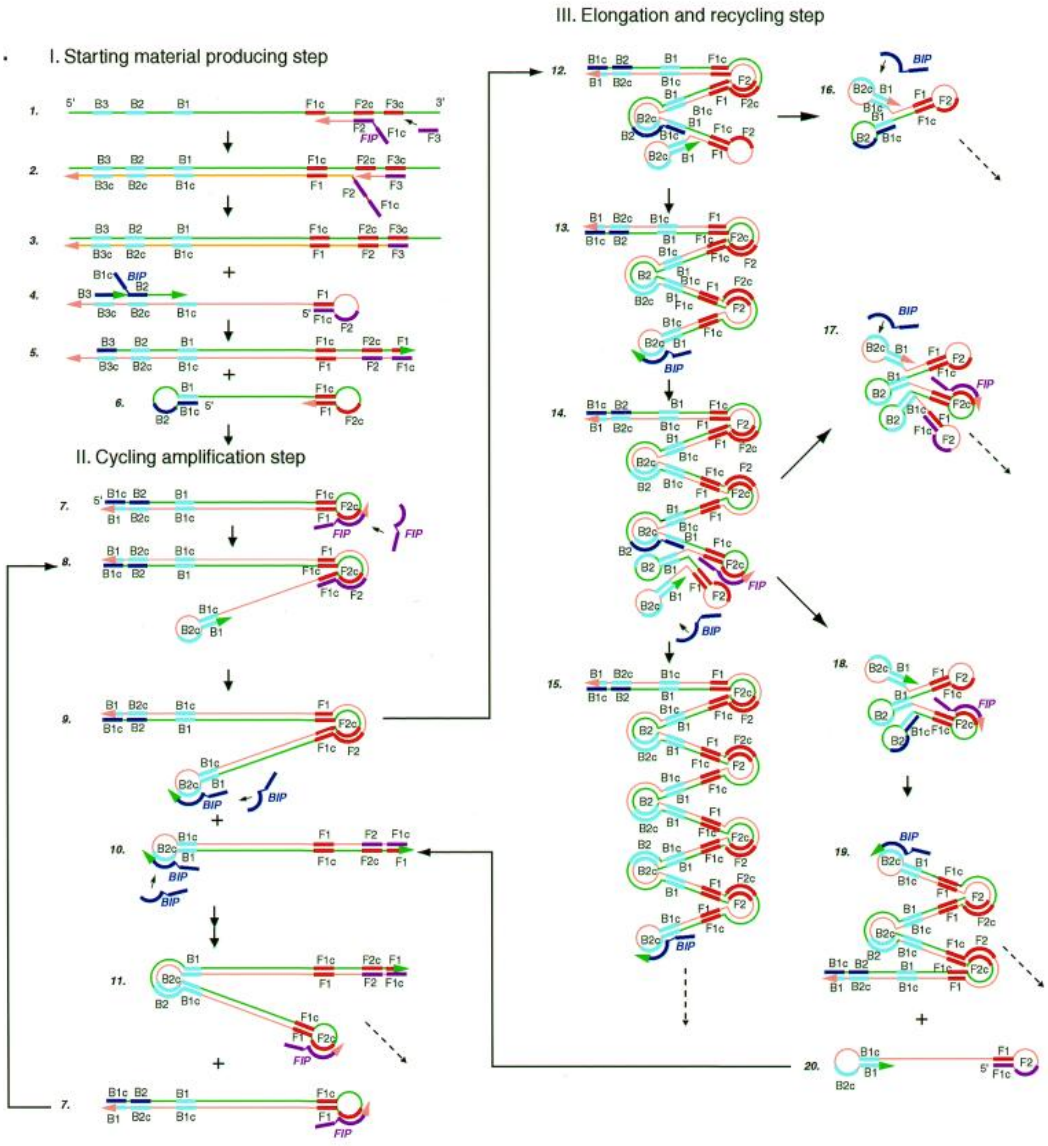


Figure 4.1 Schematic of LAMP reaction (20)

Dielectrophoresis (DEP) is a phenomenon of a non-uniform electric field which electric force exerts on a polarizable object. Surface charges of the object are induced into the polarizable object in the electric field which a positive charge on one side of the object and negative charge on another. Under a lower polarization of a surrounding medium, the electric field density is higher on the one side of the object than another side so the direction of the higher field strength exerts net force acting on the object. The dielectrophoresis can be categorized into two classes, i.e., positive and negative dielectrophoresis. In positive dielectrophoresis, a particle has the higher polarizability than the surrounding medium and then is pushed towards the higher electric field region. In the other hand, the suspended particle has the smaller polarizability than the surrounding medium, the particle is pushed toward to the weaker electric field region which is called negative DEP (40). In the theory and calculation, dielectrophoresis force is exerted on different kinds of particles with different characteristics of dielectrophoresis force depending on the shape and the size of the particles and the sort of the surrounding medium as well as the design and dimension of electrode and chamber of solution filled in the electrical field in micro-fluidic detection system. Theoretical calculation should be re-calculated for each type of particle to be measured. Dielectrophoresis force calculation has various results on each type of particle (161).

In the present study, dielectrophoretic impedance measurement method was applied for detection of 189 bp-PCR products and LAMP products which containing various sizes of double-stranded DNA. Additionally, a fluorescence microscopy was applied to observe DNA captured on microelectrodes of the device.

4.3 Materials and Methods

4.3.1 Preparation of PCR product for DEPIM

PCR primers were designed for amplification of pUC19 plasmid DNA with an amplicon size of 189 bp. Primer3 Plus bioinformatics software used for the primer design was available at http://primer3.source forge.net/ (162). The selected forward primer was 5’-ATAATACCGCGCCACATAGC-3’ and the reverse primer

was 5'-TTTGCCTTCCTGTTTTTGCT-3'. PCR was carried out in a mixture (30 μL) containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 μM of deoxynucleotide triphosphates (dNTP), 1 U of Takara EX Taq™ DNA polymerase (Takara-bio, Japan), 0.3 μM of primers and 20 ng of pUC19 plasmid DNA. The amplification profile was as follows: 95°C for 3 min; 30 cycles of 95°C for 15 s, 52-63°C for 30 sec, 72°C for 1 min; final extension at 72°C for 5 min. PCR products of each annealing temperature was analyzed by agarose gel electrophoresis and visualized under illuminator (Maestrogen, Taiwan) after staining with SYBR safe dye. PCR product generated from an optimal annealing temperature (at 58.3°C) was purified from agarose gel using Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Column Kit (Bio-Rad, USA) according to manufacturer's protocol. Briefly, 189 bp-band in agarose gel was excised by razor blade and the trimmed gel slice was put in Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Column. After freezing at -20°C for 5 min, the column was spun at 13,000x *g* for 3 min at room temperature. Then, DNA was cleaned up with MiniElute® Reaction Clean up Kit (Qiagen, USA) according to manufacturer's protocol. Briefly, DNA sample was added with 5 volumes of buffer PBI and applied in a spin column. The spin column was placed in 2 mL-collection tube and then centrifuged at 10,000x *g*, room temperature, for 1 min. The column containing DNA was washed with 750 μL of buffer PE and DNA was eluted with distilled water. Collected DNA (PCR product) was confirmed by running on agarose gel electrophoresis. The 189 bp-DNA was used for analysis by DEPIM equipment.

4.3.2 Preparation of LAMP product for DEPIM

Four specific primers including Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Outer Primer (F3) and Backward Outer Primer (B3) were designed using Primer Explorer Version 4 (http://primerexplorer.jp/e/) targeting pUC19 plasmid DNA. The sequences of primers were F3: 5'-CCATTGCTACAGGCATCGT-3'; B3: 5'-GCAGTGCTGCCATAACCAT-3'; FIP: 5'-TGGGGGATCATGTAACTCGCCTTTTTGTGTCACGCTCGTCGTTTG-3'; and BIP: 5'-GCGGTTAGCTCCTTCGGTCCTTTTTGATAACACTGCGGCCAAC-3'. The optimum temperature for amplification of LAMP reaction was determined at 60, 62, 65, 67 and 68ºC for 60 min followed by an analysis of the products by gel

electrophoresis and visualization under transluminator after staining with SYBR safe. The reaction mixture contained 2 μM each of inner primers FIP and BIP, 0.2 μM each of outer primer F3 and B3, 1.4 mM of dNTP (Takara-bio, Japan), 0.6 M of betaine (Sigma-Aldrich, USA), 6 mM of $MgSO_4$, 8 U of *Bst* DNA polymerase, 1X of the supplied buffer and 2 μL of Template DNA in a final volume of 25 μL. The solution of LAMP product was used for analysis by DEPIM equipment.

4.3.3 DEPIM equipment

The DEPIM apparatus consisted of three major parts: a castellated pattern microelectrode, an AC voltage source and an impedance analyzer as shown in Figure 4.2. An interdigitated microelectrode of a chrome thin film was patterned on a glass substrate by photolithography technique. The electrode had a castle-wall pattern in order to form high and low electric field regions periodically. Each electrode finger was 5 mm long and was separated by minimum clearance of 5 μm. The castellation was squares with sides of 50 μm. The 20 electrode fingers formed 19 castellated gaps. These microelectrodes were surrounded by a silicon rubber spacer to form a chamber in which 15 μl of a liquid was stored. Sinusoidal ac voltage was generated by a function generator and applied to the electrode system. Electrode impedance measurements were carried out using a DSP lock-in amplifier (Model 7280, Perkin-Elmer Instruments, USA). The impedance measurement apparatus was controlled by a personal computer and the computer also functioned as a data recorder and analyzer. Dielectrophoresis was visually observed DNA trapping by using a fluorescence microscope and a CCD digital camera (COOLPIX 990, Nikon, Japan).

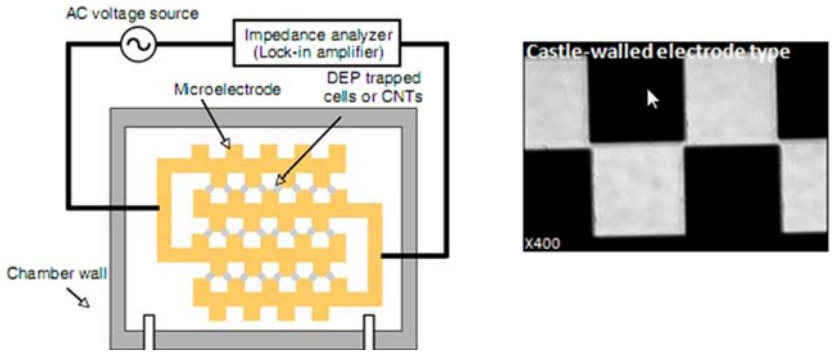


Figure 4.2 A schematic diagram of DEPIM equipment and castle-walled electrodes.

4.3.4 Experimental procedures

For an observation of DNA trapping to the microelectrode, PCR product (189 bp-DNA) a concentration of 20 μM for 10 μL and LAMP products with were stained with SYTO9 (Invitrogen, Japan) and 20 μL of the suspension was filled into the microelectrode chamber. Various frequency (150 kHz-2 MHz) and AC voltage (5V, 10V, 15V and 20V) were applied to the DEPIM system for 30 s which energizes the interdigitated eletrode to generate positive DEP force. During these processes, visual observation of DNA trapping and DEPIM inspection was conducted simultaneously.

For DEPIM detection of DNA trapping, PCR product and LAMP products without a process of SYTO9 staining were loaded into the microelectrode chamber and then AC voltage of 150 kHz frequency and 5V amplitude was applied for 350 s. During these processes, DEPIM inspection resulted in an electrode conductance was continuously monitored and recorded with a sampling internal of 20 ms.

4.4 Results and Discussion

4.4.1 Preparation of PCR product for DEPIM

The determination of optimum annealing temperature for PCR was carried out at 52.0, 52.2, 52.8, 53.7, 54.9, 56.4, 58.3, 59.9, 61.1 and 62.2^{o}C for 60 min. All

temperatures resulted in clear and strong bands as shown in Figure 4.3, however annealing temperature of 58.3°C was chosen. After the PCR product was extracted for agarose gel, the DNA was run on agarose gel electrophoresis to confirm a size of 189 bp as shown in Figure 4.4. The 189 bp-DNA was further used for defining a DNA trapping on dielectrophoresis electrode under fluorescence microscopy.

4.4.2 Preparation of LAMP product for DEPIM

The determination of optimum temperature for LAMP reaction was carried out at 60.0, 62.1, 64.6, 66.6 and 68.0ºC for 60 min (Figure 4.5). At temperatures of 66.6ºC was arbitrarily chosen as the assay temperature.

4.4.3 Observation of 189 bp-DNA trapping to microelectrode

The 189 bp-DNA at a concentration of 1 mM was stained with 2 mM of SYTO9 and then filled into the DEP chamber. The DEP observations were made at electric field frequency of 2 MHz with various currents of 5V, 10V, 15V and 20V for 2 min. DNA trapped in the gap between two castellated electrodes was not observed in these experiments for all voltages applied. Photographs of DEP castled-wall electrodes without DNA trapping was shown in Figure 4.6. Generally, increasing of a frequency for DNA trapping to DEP electrodes could slightly decreased a polarizability of DNA fragment. With an exception for applying with 1 MHz frequency, polarizability was increased in most DNA fragments (164).

4.4.4 Observation of LAMP product trapping to microelectrode

The LAMP product was diluted with distilled water to a 1:10 dilution and 2 mM of SYTO9 dye was added with an equal volume. The DEP observations were made at electric field frequencies of 150 kHz-2 MHz with various currents of 1V-20V for 1 min. DNA trapped in the gap between two castellated electrodes was not observed in these experiments for all frequencies and currents applied. A photograph of DEP castled-wall electrodes was shown in Figure 4.7. DNA trapping on electrodes was not observed, however, DNA precipitation that moving independently in the chamber was noticed.

4.4.5 DEPIM measurement using PCR product of 189 bp-DNA

The 189 bp-DNA stained with SYTO9 (2 mM dye:1 mM DNA) was filled into DEP chamber and applied with an ac voltage of 150 kHz frequency and 5 V amplitude for 5 min. The conductance G_T is defined as the electrode conductance from the initial value of the DEPIM. The electrical conductance of the microelectrode increased to about 6.3 μS during DEP process (Figure 4.8). While the conductance of negative control which was only SYTO9 dye was slightly increased up to 4 μS. The data showed a possibility of using DEPIM for a detection of PCR product.

4.4.6 DEPIM measurement with undiluted LAMP product

After performing a LAMP reaction, 10 μL of LAMP positive reaction and 10 μL of LAMP negative reaction without staining with SYTO9 dye were used to detect by DEPIM. The 150 kHz and 5 voltages of current were applied into measurement system. The LAMP positive reaction (Figure 4.9) gave significantly higher conductance values (about 40 times) than those of the LAMP negative reaction. The data showed a possibility of using DEPIM for a detection of LAMP product. However, a high temperature of DEP chamber was detected when applied with a current of 5 V. The high temperature may cause a bubble formation nearby an electrode which may interfere with the trapping of molecules on the electrode.

4.4.7 Effect of diluents, currents and temperatures on DEPIM

The conductivity of a medium or a diluent is an important factor that affects a positive or negative charge of DEPIM. For measuring of small molecules, such as DNA, a low conductivity of medium is needed because a high conductivity of the system can produce heat in the measuring chamber when a current is applied to the electrode. Two diluents for LAMP product were studied including distilled water and ethanol. Both diluents were filled directly on the electrode chamber of DEPIM and then a 150 kHz and 5 V was applied. DEPIM results of distilled water (Figure 4.10 A) showed an increasing conductance up to 6 μS at 300 s. While the conductivity of ethanol sharply increased up to 110 μS after 75 sec and then suddenly dropped to less than zero because of an vaporization of ethanol (Figure 4.10 B). The increase of

temperature depends on the electrical field strength applied on the electrode. The current of 5 V was generated heat in the microelectrode chamber.

The effect of an electric current was studied by filling distilled water into a DEP chamber and applying a current of 3 V and 5 V with 150 kHz to compare their conductivity. When a high voltage (5V) was applied on the electrode resulting in a higher conductivity with time-dependence (up to 13 μS at 300 s) of the suspension medium as shown in Figure 4.11 A. While a lower conductivity (below 1 μS) was observed at a 3 V of current applied as shown in Figure 4.11 B. The lower conductivity of a base line measurement is preferred for a DEPIM experiment.

To control of heat generated during DEPIM when using ethanol as a diluent, the electrodes were placed on ice after applying ethanol into the chamber. When the measurement was performed at room temperature, the unsteady conductance was noticed in the first 150 sec (Figure 4.12 A). Contrastly, a constant decreasing of conductance values was observed when the electrode was placed on ice (Figure 4.12 B).

4.4.8 DEPIM measurement with diluted LAMP product

After performing a LAMP reaction, LAMP positive reaction and LAMP negative reaction were diluted with distilled water (1:10 dilution) and then filled into an electrode chamber for DEPIM. The 150 kHz and 3 voltages of current were applied into the measurement system. The LAMP positive product gave very high conductance values to 100 μS at 50 s and to 130 μS at 300 s (Figure 4.13). However, a conductivity of LAMP negative sample was as low as 7 μS at 200 sec (Figure 4.9).
The data also showed a possibility of using DEPIM for a detection of diluted LAMP product. In general, the conductivity of a suspension medium prepared for DEPIM should be about 20 μS/cm. However, the conductivity of 1:10 dilution of LAMP produce was still very high as in mS/cm. More dilution (1:3600) of the samples was performed in the next experiments.

To lower conductivity for DEPIM of LAMP product to reach the conductivity value of about ~20 μS/cm, making 1:3600 dilution with distilled water of the product was performed. After filling the dilutions into microelectrode chamber, the 150 kHz and 3 V of current was applied into measurement system. The LAMP

positive product resulted in low conductance values to less than 3.5 μS at 300 s (Figure 4.14) which were lower than a conductivity of LAMP negative sample that was about 6 μS at 300 s. This experiment showed that more diluted LAMP positive product could not be detected by the DEPIM system.

When detecting LAMP product by diluting with ethanol, LAMP positive and negative reaction were diluted with ethanol (1:1000 dilution) to get the conductivity value of about ~20 μS/cm. The LAMP positive reaction was observed with increasing conductivity reaching up to about 11 μS at 260 s as shown in Figure 4.15. While the conductivity of the LAMP negative reaction was not exceed 0.8 μS. The data also showed a possibility of using DEPIM for a detection of diluted LAMP product by using ethanol as a diluent.

4.5 Conclusion

In this study, the DEPIM method was applied for detection of PCR and LAMP products by measuring an increment of conductance. In an AC electric field of 150 kHz, the conductance increased when applying the undiluted PCR product (187 base pair). After optimizing conditions for detecting LAMP product, only undiluted and ten-fold diluted of the LAMP products demonstrated conductance increment when compared with the controls. This preliminary study showed the possibility of applying DEPIM method for detection of PCR and LAMP products.

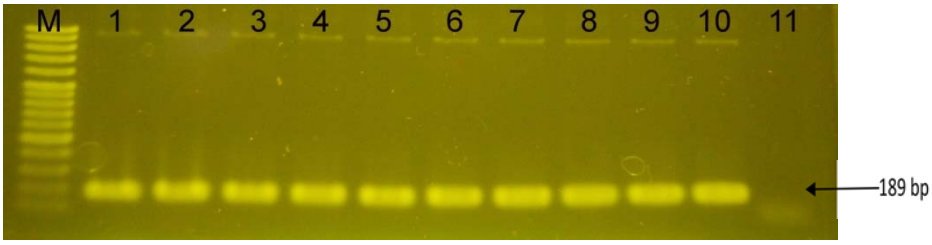


Figure 4.3 Optimization of annealing temperatures for PCR. Each lane gave the same amplified 189 bp-DNA while no band was observed in negative control. (M=100 bp DNA ladder, lane 1=52.0°C, lane 2= 52.2°C, lane 3=52.8°C, lane 4=53.7°C, lane 5=54.9°C, lane 6=56.4°C, lane 7=58.3°C, lane 8=59.9°C, lane 9=61.1°C, lane 10=62.2°C, and lane 11= negative control). The 1% agarose gel electrophoresis was performed with 100 V applying for 30 min.

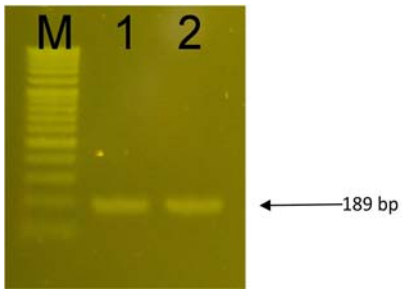


Figure 4.4 The 189 bp-DNA band extracted from agarose gel. The 1% agarose gel electrophoresis was performed with 100 V applying for 30 min.

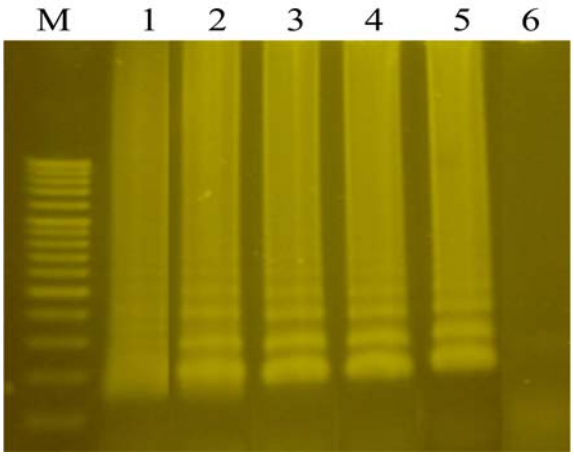


Figure 4.5 Optimization of temperatures for LAMP reaction using pUC19 plasmid DNA. Lane M: molecular marker; lane 1-5 at temperatures of 60.0, 62.1, 64.6, 66.6 and 68.0°C, respectively; and lane 6: no-template control. The 1% agarose gel electrophoresis was performed with 100 V applying for 30 min.

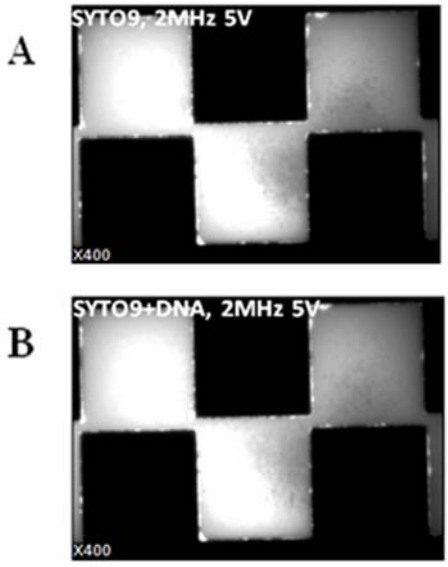


Figure 4.6 Photographs of DEP castled-wall electrodes filled without (A) and with (A) 189bp-DNA that stained with SYTO9. The DEPIM experiments were conducted at 2 MHz and 5V for 2 min.

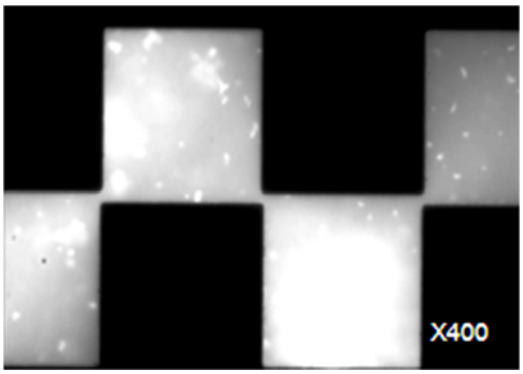


Figure 4.7 Photographs of DEP castled-wall electrodes filled with LAMP product which stained with SYTO9.

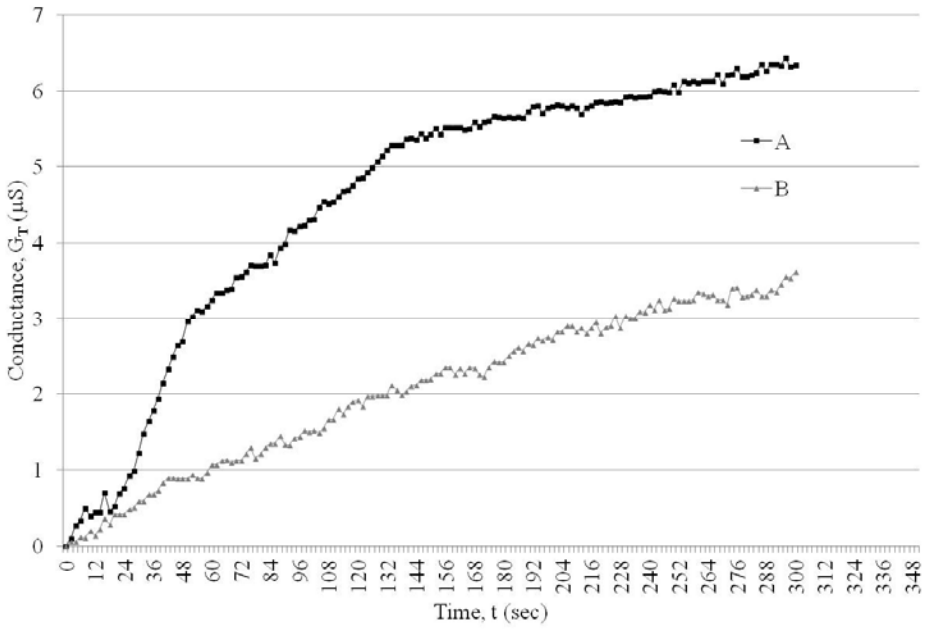


Figure 4.8 DEPIM results of 189 bp-DNA stained with SYTO9 (A) and only SYTO9 dye (B) as negative control, when applying 150 kHz and 5 V.

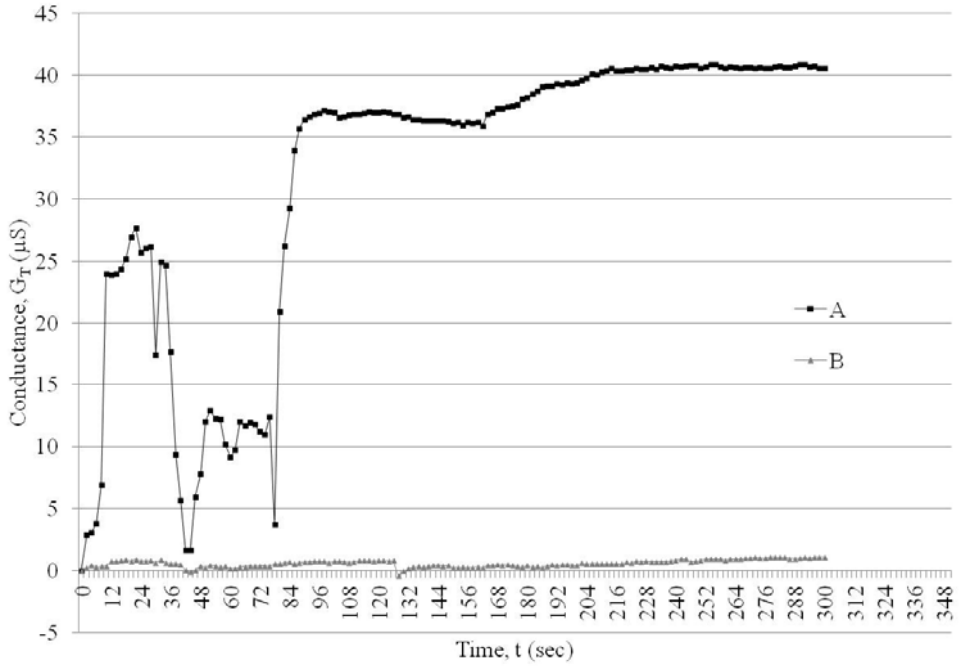


Figure 4.9 DEPIM results of undiluted LAMP product (A) and negative control (B) when applied with 150 kHz and 5 V.

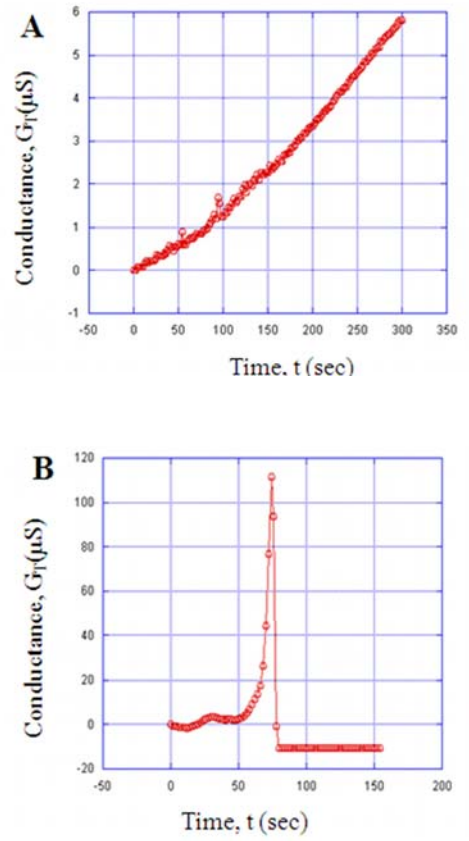


Figure 4.10 DEPIM results of distilled water (A) and ethanol (B) when applied with 150 kHz and 5 V.

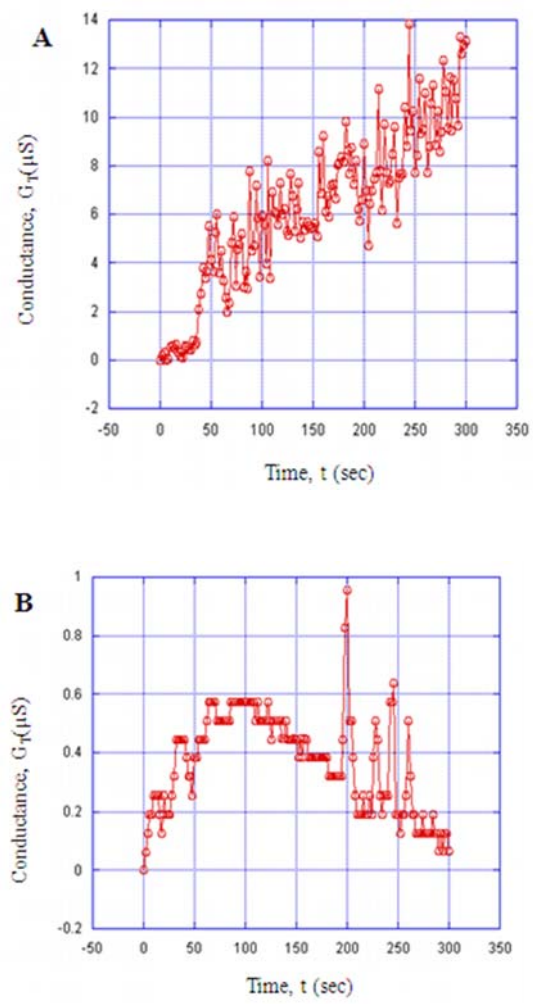


Figure 4.11 Using distilled water as a diluent running DEPIM at room temperature with a current of 5 V (A) and 3 V (B) when applied with 150 kHz.

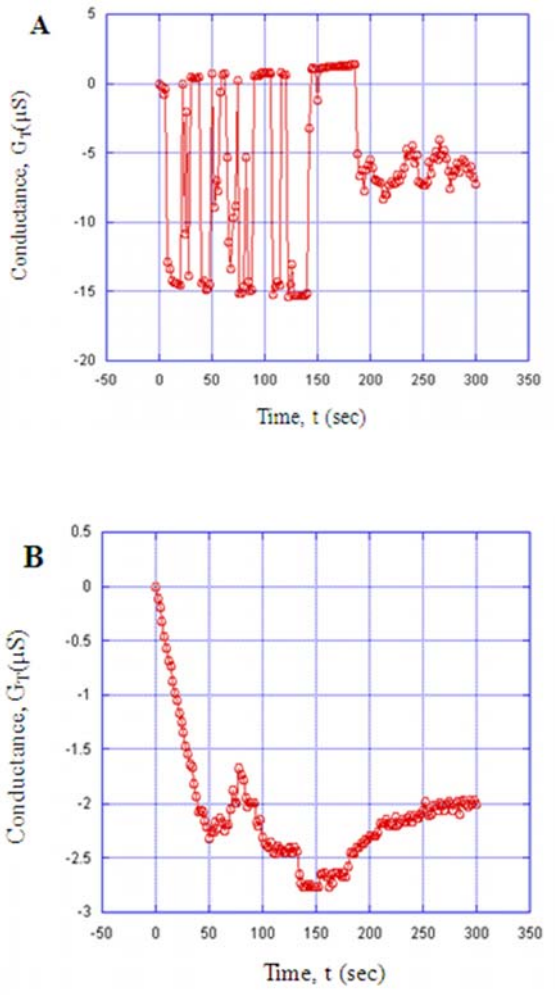


Figure 4.12 Using ethanol as a diluent for running DEPIM at room temperature (A) and on ice (B) when applied with 150 kHz and 3 V.

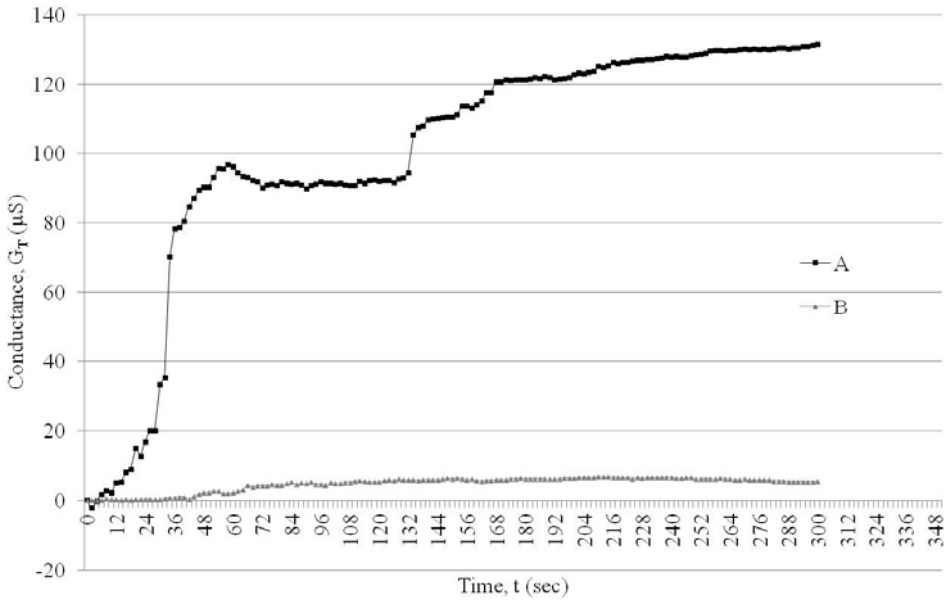


Figure 4.13 DEPIM results of LAMP product. Diluted 1:10 with distilled water (A) and negative control (B) when applied with 150 kHz and 3 V.

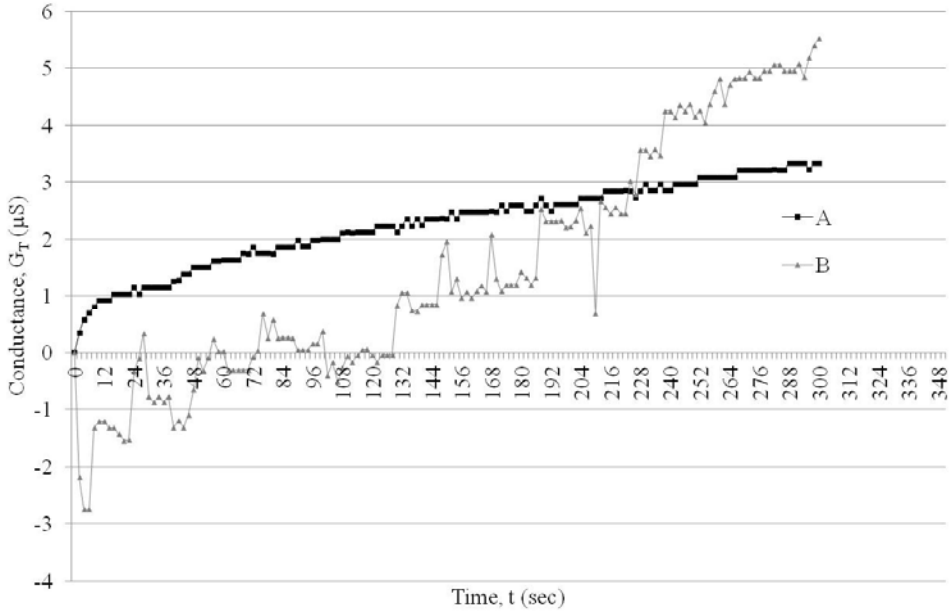


Figure 4.14 DEPIM results of LAMP product. Diluted 1:3600 with distilled water (A) and negative control (B) when applied with 150 kHz and 3 V.

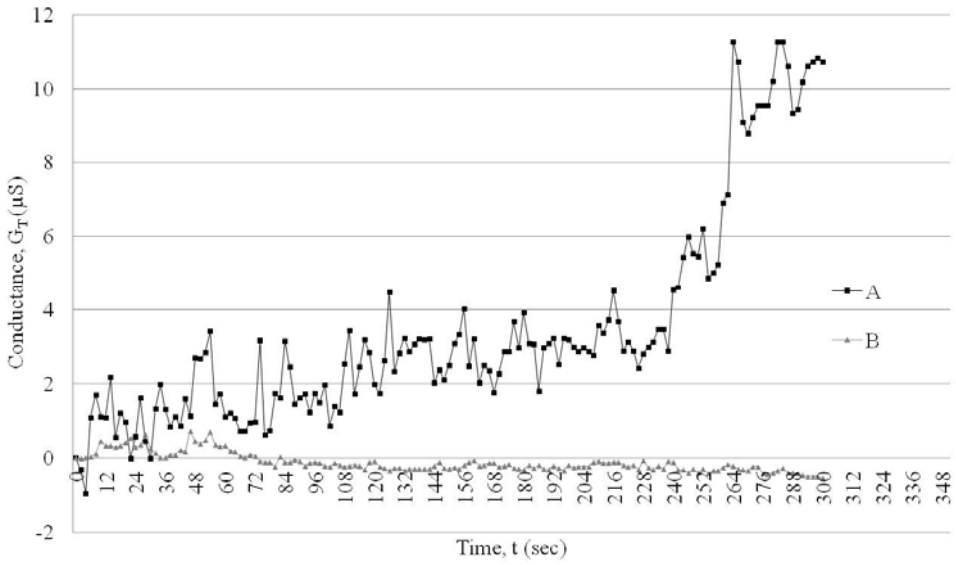


Figure 4.15 DEPIM results of LAMP product. Diluted 1:1000 with ethanol (A) and negative control (B) when applied with 150 kHz and 3 V.

CHAPTER V
CONCLUSION AND FUTURE PERSPECTIVES

The detection and identification of thermophilic *Campylobacter* isolated from clinical specimens and food samples is important for microbiological laboratories. The realiable and rapid results are needed for appropriate treatments in gastroenteritis patients, especially in children cases, and for investigation of an outbreak or sporadic diseases. *Campylobacter* spp. is important foodborne pathogens causing acute enteritis worldwide. Poultry are considered as a major reservoir for *Campylobacter* transmission to human because a consumption of contaminated raw chicken products. The detection of the organisms from chicken and their products in farms, slaughter plants, retailed markets and nearby environments is essential for biosecurity and food safety. Conventional standard method is culture-based technique which has several drawbacks including time-consuming and labor-intensive. Nowadays, molecular methods based on PCR technology for *Campylobacter* detection are commercially available, however an expensive analyzer is required for the assay manipulation. A simple, practical, rapid and reliable method is still vital to develop for using in developing countries and for on-fields investigation.

In this study, a magneto-PCR-enzyme linked gene assay was developed for detection of thermophilic *Campylobacter* species from pure culture and chicken skin samples. Magnetic nanoparticles are benefit for separating target DNA away from interfering substances of PCR reaction in sample matrices. Forward and reverse primers were designed to specific for 16S rRNA of thermophilic *Campylobacter*. The forward primers immobilized on magnetic particles captured *Campylobacter* DNA to be amplified by PCR reaction. The amplicons attached with the magnetic nanoparticles were collected by an external magnetic field and subjected to reaction with streptavidin-horseradish peroxidase and its substrate. The developed color products were measured easily by spectrophotometer. The developed method had high specificity and high sensitivity with one picogram detection of *Campylobacter* DNA.

The method was successfully applied for detection of *Campylobacter* in naturally contaminated chicken skin samples. All positive results were comparable to the results that were analyzed by the conventional cultural method and conventional PCR assay. Application of the magneto-PCR-enzyme linked gene technique should be studied more on various types of food samples and clinical specimens. Additionally, analytical method validation should be performed for ensuring a specified performance of the assay.

Another work of this thesis was preliminary application of the dielectrophoretic impedance measurement (DEPIM) method for detection of PCR and LAMP products. Dielectrophoresis is the electrokinetic motion of the polarized particles in spatially non-unifiorm electric fields and its applications on DNA separation and concentration are currently combined with molecular detection systems. The DEPIM method measured a conductance increment after applying an AC electric fields on the castle-wall type microelectrodes. DEP-trapping process of double-stranded DNA for PCR or LAMP products was visualized by a fluorescence microscopy. However, this study could not observe the DNA trapping on the electrodes. Nevertheless, DEPIM results represented as a conductance values were observed with both PCR and LAMP products. This preliminary study showed the possibility of applying DEPIM method for detection of PCR and LAMP products. Optimization of various conditions including electrical frequencies and currents for DEPIM method needed to be performed intensively for further applications of DNA detection and separation.

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APPENDIX

MEDIA AND BUFFER PREPARATION

Bolton broth

Bolton broth was prepared according to their formulation. Bolton broth (Oxoid LTD., Hampshire, England) was prepared according to the manufacturer's procedure with some modifications. Bolton broth (13.8 g) was added to 500 mL of distilled water and sterilized by autoclaving at 121°C for 15 min. One vial of Bolton broth selective supplement (SR0183; Oxoid) which reconstituted as directed was aseptically added into the broth. The broth was distributed into sterile screw top containers.

Charcoal cefoperazone deoxycholate agar

Campylobacter blood-free selective agar base (charcoal desoxycholate agar; CDA; Oxoid) was prepared following the manufacturer's instruction. *Campylobacter* blood-free selective agar base (22.75 g) was suspended in 500 mLof distilled water and sterilized by autoclaving at 121°C for 15 min. After cooling to 50°C, CCDA selective supplement (SR0155; Oxoid) which reconstituted as directed was aseptically added into the medium. The medium was poured into sterile petri dishes and stored in the dark for up to 2 weeks at 2-8°C.

Tris-HCl stock solution (1.0 M, pH 7.4)

Tris Base (60.57 g) was added to an 500 mL-beaker and deionized water (350 mL) was added to the beaker. A stir bar was put into the solution and left on a stir plate until completely dissolved (~1 min). The solution was measured and adjusted a pH to be 7.4 by slowly adding about 32.5 mL of 12 M HCl with a caution. Once the pH of the solution is 7.4, deionized water was added to raise the volume to 500 mL. The solution was stored in the refrigerator for up to 4 months.

PUBLICATIONS INCLUDED IN THIS DOCTORAL DISSERTATION

Papers included in this thesis

1. Magneto-PCR-Enzyme Linked Gene Assay for Detection of Thermophilic *Campylobacter* Species.
   Wuttichote Jansaento, Kulachart Jangpatarapongsa, Duangporn Polpanich, Wijit Wonglumsom.
   Manuscript in Preparation
2. Dielectrophoretic Impedance Measurement Method for Detection of PCR and LAMP Products.
   Manuscript in Preparation

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