A Novel *Campylobacter* Apparatus for the Detection of Viable Thermophilic *Campylobacter* in Chicken Meat Products

Sueptrakool Wisessombat

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Microbiology

Prince of Songkla University

2009

Thesis Title	A Novel *Campylobacter* Apparatus for the Detection of Viable Thermophilic *Campylobacter* in Chicken Meat Products
Author	Mr. Sueptrakool Wisessombat
Major Program	Microbiology

Major Advisor:

(Assoc. Prof. Dr. Supayang P. Voravuthikunchai)

Co-advisors:

(Asst. Prof. Dr. Kanokwan Kittiniyom)

(Asst. Prof. Dr. Potjanee Srimanote)

(Prof. Dr. Hazel Mitchell)

Examining Committee:

(Assoc. Prof. Dr. Nongyao Sawangjaroen) Chairperson

(Assoc. Prof. Dr. Supayang P. Voravuthikunchai)

(Asst. Prof. Dr. Kanokwan Kittiniyom)

(Asst. Prof. Dr. Potjanee Srimanote)

(Asst. Prof. Dr. Wijit Wonglumsom)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Microbiology

(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ACKNOWLEDGMENT

I dedicate this thesis and my sincerest gratitude to my supervisor, Assoc. Prof. Dr. Supayang Voravuthikunchai for continued support and guidance. Thanks for the constant help and many opportunities that have been given to me. Also thanks for all the encouragement and being at my side.

I would like to express my gratitude to my co-advisors, Asst. Prof. Dr. Kanokwan Kittiniyom, Asst. Prof. Dr. Potjanee Srimanote, and Prof. Dr. Hazel Mitchell for their continued support and kind permission for my study. I also wish to appreciate my examining committees, Asst. Prof. Dr. Wijit Wonglumsom and Assoc. Prof. Dr. Nongyao Sawangjaroen for their kindness.

I acknowledge the considerable financial support provided by the National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand (Project No. BT-B-01-NG-18-5005), Fiscal year 2007-2009 and Prince of Songkla University Graduate Fund, Fiscal year 2006-2009, Thailand.

Special thanks to my girlfriend, Nattiya Wattanakitjanukul for help and cheering me up as always. Many thanks to all Natural Products Research Center members. Finally, my sincerest gratitude goes to mother, father, and my brother for their patience, understanding and encouragement for my study.

Sueptrakool Wisessombat

ชื่อวิทยานิพนธ์ ระบบตรวจวิเคราะห์หาเชื้อ *Campylobacter* ในเนื้อไก่
ผู้เขียน นายสืบตระกูล วิเศษสมบัติ
สาขาวิชา จุลชีววิทยา
ปีการศึกษา 2552

บทคัดย่อ

เชื้อ *Campylobacter* กลุ่มที่ชอบอุณหภูมิสูง เป็นแบคทีเรียก่อโรคอุจจาระร่วงที่เป็นปัญหาสำคัญ ชนิดของเชื้อที่พบได้บ่อยในการก่อโรคในมนุษย์ ได้แก่ *C. jejuni*, *C. coli*, *C. lari,* และ *C. upsaliensis* โดยมักปนเปื้อนมาจากผลิตภัณฑ์จากสัตว์โดยเฉพาะเนื้อไก่ และผลิตภัณฑ์จากเนื้อไก่ ด้วยเหตุนี้สำหรับประเทศไทยซึ่งเป็นแหล่งผลิตเนื้อไก่รายใหญ่ในภูมิภาคเอเชียตะวันออกเฉียงใต้สำหรับส่งออกในตลาดโลก จึงมีความจำเป็นที่ต้องมีการควบคุมคุณภาพความปลอดภัยของผลิตภัณฑ์ทางจุลชีววิทยา วิธีมาตรฐานที่ใช้ในการแยกเชื้อ *Campylobacter* จากตัวอย่างอาหารเนื้อไก่นั้นคือ วิธีเพาะเลี้ยงเชื้อและตรวจสอบทางชีวเคมี แต่วิธีดังกล่าวค่อนข้างซับซ้อน ต้องใช้บุคลากรที่มีประสบการณ์ รวมทั้งมีค่าใช้จ่ายสูง และใช้ระยะเวลานาน ดังนั้นในการศึกษานี้จึงมีจุดประสงค์ที่จะพัฒนาระบบที่รวดเร็ว มีประสิทธิภาพในการตรวจแยกเชื้อ *Campylobacter* จากเนื้อไก่ อุปกรณ์ตรวจแยกเชื้อที่ได้พัฒนาขึ้นนั้น อาศัยหลักการที่เชื้อ *Campylobacter* มีชีวิตสามารถเคลื่อนที่ผ่านรูของแผ่นกรองขนาด 0.45 ไมโครเมตร ซึ่งเคลือบด้วยอาหารหนืดได้ ซึ่งเป็นขนาดของรูที่แบคทีเรียชนิดอื่นไม่สามารถเคลื่อนที่ผ่านได้ จากการศึกษาเชื้อ *Campylobacter* สายพันธุ์มาตรฐาน จำนวน 7 สายพันธุ์ พบว่าอุปกรณ์แยกเชื้อชนิดใหม่นี้ สามารถตรวจแยกเชื้อ *Campylobacter* มีชีวิตที่ปนเปื้อนในเนื้อไก่ได้ ถึงแม้จะมีจำนวนเซลล์แบคทีเรียที่มีชีวิตเพียง 10 เซลล์ต่อเนื้อไก่ 1 กรัม และมีความจำเพาะต่อเชื้อ *Campylobacter* เป็น 100%

การบ่งชี้ชนิดของเชื้อ *Campylobacter* ที่มีบทบาทสำคัญในการก่อโรค ได้แก่ *C. jejuni* และ *C. coli* นั้น ใช้การทดสอบ hippurate hydrolysis และความไวต่อยาปฏิชีวนะ ซึ่งอาจให้ผลการทดสอบที่ไม่ถูกต้อง ดังนั้นการศึกษาในครั้งนี้จึงได้พัฒนาวิธีการบ่งชี้ชนิดของเชื้อ *Campylobacter* ด้วยเทคนิค multiplex PCR ซึ่งเป็นเทคนิคที่มีความจำเพาะสูง ทำให้สามารถบ่งชี้ชนิดของเชื้อได้รวดเร็ว และมีประสิทธิภาพยิ่งขึ้น จากการศึกษาประสิทธิภาพของชุดตรวจวินิจฉัย multiplex PCR ที่ได้พัฒนาขึ้นกับวิธีมาตรฐานในการบ่งชี้ชนิดของเชื้อนั้น พบว่าชุดตรวจวินิจฉัย multiplex PCR ตรวจพบดีเอ็นเอที่ความเข้มข้น 100 นาโนกรัม หรือ 2×10^{5} ของเซลล์แบคทีเรียมีชีวิต และเมื่อบ่งชี้ชนิดของเชื้อด้วยชุดตรวจวินิจฉัย multiplex PCR กับตัวอย่างเชื้อ *Campylobacter* ทั้งหมด 78 ตัวอย่าง พบว่า 55 ตัวอย่าง คิดเป็น 70.5% แสดงผลเป็นเชื้อ *C. jejuni* และ 18 ตัวอย่าง คิดเป็น 23.0% คือเชื้อ *C. coli* ในขณะที่ 5 ตัวอย่าง คิดเป็น 6.41% เป็นเชื้อผสมของ *C. jejuni* และ *C. coli* และตรวจพบจีน *cdtB* ได้ในเชื้อ *C. jejuni* และ *C. coli* คิดเป็น 100% และ 38.9% ตามลำดับ เมื่อเปรียบเทียบกับวิธีมาตรฐานที่ใช้บ่งชี้ชนิดของเชื้อ พบว่ามีทั้ง ผลบวกปลอม และผลลบปลอม คิดเป็น 6.41% และ 3.85% ตามลำดับ

อย่างไรก็ตามพบว่าการใช้อุปกรณ์แยกเชื้อชนิดใหม่นี้ ในการตรวจแยกเชื้อจากเนื้อไก่นั้น ต้องใช้เวลาบ่มตัวอย่างอาหารอย่างน้อย 18 ชั่วโมง จึงจะตรวจพบเชื้อมีชีวิตได้อย่างน้อย 2×10^5 เซลล์ ดังนั้นจึงได้ศึกษาผลของ chemotactic ต่อความสามารถในการชักนำการเคลื่อนที่ของเชื้อ *Campylobacter* เพื่อจะลดเวลาที่ใช้ในการบ่มแยกเชื้อจากเนื้อไก่ได้ จากผลการศึกษาพบว่าสารประกอบของ mucin และ bile ที่ความเข้มข้น 1%, 5%, และ 10% ต่างมีผลชักนำการเคลื่อนที่ของเชื้อ *Campylobacter* ได้ โดยสามารถแยกจำนวนเชื้อมีชีวิตได้มากกว่าอย่างมีนัยสำคัญ ใช้เวลาในการบ่มแยกเชื้อเพียง 12 ชั่วโมงเท่านั้น และพบว่าเซลล์ที่บาดเจ็บของเชื้อ *Campylobacter* นั้นตอบสนองต่อสารประกอบของ mucin และ bile ลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับเซลล์ปรกติ

เมื่อนำอุปกรณ์ตรวจแยกเชื้อที่ประดิษฐ์มาใช้ควบคู่กับชุดตรวจวินิจฉัย multiplex PCR ที่ได้พัฒนาในการตรวจแยกเชื้อ *Campylobacter* จากตัวอย่างเนื้อไก่ พบว่าจากจำนวนตัวอย่างเนื้อไก่ทั้งหมด 305 ตัวอย่าง อุปกรณ์ตรวจแยกเชื้อชนิดใหม่ตรวจพบว่ามีเชื้อ *Campylobacter* ปนเปื้อนจำนวน 31 ตัวอย่าง คิดเป็น 10.16% โดย 28 ตัวอย่าง คิดเป็น 9.18% ปนเปื้อนเชื้อ *C. coli* และ 3 ตัวอย่าง คิดเป็น 0.98% ปนเปื้อนเชื้อ *C. jejuni* ในขณะที่วิธีมาตรฐานนั้น ตรวจพบตัวอย่างอาหารปนเปื้อนเชื้อ *Campylobacter* จำนวน 14 ตัวอย่าง คิดเป็น 4.59% โดยพบว่าเป็นเชื้อ *C. coli* ทั้งหมด จากการศึกษาแสดงให้เห็นว่า การใช้อุปกรณ์ตรวจแยกเชื้อชนิดใหม่ที่ได้พัฒนาขึ้นร่วมกับชุดตรวจวินิจฉัย multiplex PCR สามารถเพิ่มโอกาสในการตรวจพบเชื้อได้มากกว่า และมีความจำเพาะสูงกว่าวิธีมาตรฐาน

จากการศึกษาเพิ่มเติมยังพบว่าอุปกรณ์นี้แยกเชื้อที่มีชีวิตของ *C. concisus* และ *Helicobacter pylori* ซึ่งเป็นแบคทีเรียที่เป็นสาเหตุของอาการลำไส้อักเสบในมนุษย์ได้ โดยสามารถตรวจพบแบคทีเรียได้ที่จำนวนเซลล์อย่างน้อย 10^2 cfu/ml และเมื่อนำไปประยุกต์ใช้ในการตรวจแยกเชื้อแบคทีเรียดังกล่าวจากชิ้นเนื้อตัวอย่าง (gastric biopsy) ของผู้ป่วย จำนวน 5 ตัวอย่าง เปรียบเทียบกับวิธีมาตรฐาน พบว่าตรวจไม่พบเชื้อจากทั้งสองวิธี อย่างไรก็ตามยังต้องการจำนวนตัวอย่างชิ้นเนื้อเพิ่มมากขึ้นเพื่อยืนยันผลการศึกษาต่อไป

กล่าวได้ว่าอุปกรณ์ตรวจแยกเชื้อที่ประดิษฐ์มีข้อดีคือ ทำให้สามารถแยกเชื้อ *Campylobacter* ที่มีชีวิตจากเชื้อชนิดอื่นที่ปนเปื้อนในอาหารได้ ในขณะที่การตรวจวินิจฉัยบ่งชี้ชนิดของเชื้อทำได้รวดเร็วยิ่งขึ้นด้วยเทคนิค multiplex PCR ที่ได้พัฒนาควบคู่กัน โดยสามารถบ่งชี้ชนิดของเชื้อได้จากทั้งดีเอ็นเอ และเซลล์แบคทีเรีย และตรวจหาจีน *cdtB* ซึ่งมีส่วนในการเกิดโรคได้ อันจะสามารถใช้ข้อมูลนี้ให้เป็นประโยชน์ในทางระบาดวิทยาต่อไป อีกทั้งเป็นวิธีที่ง่าย มีราคาถูก มีความไว ความจำเพาะสูง และใช้ระยะเวลาสั้นภายใน 1 วัน โดยเป็นขั้นตอนการแยกเชื้อ 18 ชั่วโมง และบ่งชี้ชนิดของเชื้อ 6 ชั่วโมง ดังนั้นเชื่อว่าหากสามารถนำระบบการตรวจวิเคราะห์เชื้อนี้ไปใช้จริงจะช่วยรับรองคุณภาพของสินค้าเนื้อไก่ส่งออก และสร้างความเข้มแข็งให้กับธุรกิจนี้เป็นอย่างมาก

Thesis Title	A novel *Campylobacter* Apparatus for the Detection of Viable Thermophilic *Campylobacter* in Chicken Meat Products
Author	Mr. Sueptrakool Wisessombat
Major Program	Microbiology
Academic Year	2009

Abstract

Thermophilic *Campylobacter* species, *C. jejuni C. coli*, *C. lari*, and *C. upsaliensis* are most frequently isolated from diarrhoeal patients. Poultry have been recognized as the primary reservoir of *Campylobacter* and play an important role in the transmission of *Campylobacter* enteritis to humans. Thailand is one of the main poultry producing countries in South East Asia and its exported products are obviously of economic importance. Microbiological quality controls throughout the food chain production are needed. Conventional procedures for isolation of thermophilic *Campylobacter* from chicken meat samples are complex, labour intensive, and time-consuming.

The objective of this study was to create a novel *Campylobacter* culturing apparatus. The main concept of the device was based on the ability of *Campylobacter* to pass through a 0.45 µm pore size filter in viscous media. Preliminary study demonstrated that only viable *Campylobacter* moved through the membrane filter and could multiply in the enrichment culture. In addition, seven reference *Campylobacter* strains demonstrated that sensitivity and specificity of the apparatus were 10 cfu/g and 100%, respectively.

Differentiation between important species, *C. jejuni* and *C. coli* has been performed by hippurate hydrolysis and antimicrobial sensitivity tests. However, there could be errors in identification since some hippurate hydrolysis-negative *C. jejuni* and a number of nalidixic acid-resistant *C. jejuni* have been reported. The objective of the present investigation was to establish a multiplex polymerase chain reaction (PCR) for the identification of these four species and compare with conventional cultural methods. I also evaluated different DNA template preparation methods to achieve high detection rate of *Campylobacter*.

Detection limit was 100 ng DNA or 2×10^5 cfu whole-cell suspension. The multiplex PCR was applied for the direct detection and differentiation of *Campylobacter* species in 33 human and 45 chicken caeca isolates. Of the 78 specimens evaluated by the multiplex PCR, 55 (70.5%) were identified as *C. jejuni*, 18 (23.0%) as *C. coli* and 5 (6.41%) as a mixed infection with both species. Comparison of hippurate test and multiplex PCR demonstrated 5 (6.41%) isolates with false-positive hippurate enzymic activity and 3 (3.85%) with false-negative activity. *cdtB* gene was detected in 100% and 38.9% of *C. jejuni* and *C. coli*, respectively. This multiplex PCR was found to be rapid, easy to perform and had a high sensitivity and specificity, even with mixed cultures. The system is useful for the detection of the presence of *cdtB* gene which is responsible for toxin activity in *Campylobacter*.

Chemotaxis is the movement of an organism towards or away from a chemical stimulus. Since the use of the novel apparatus for isolation of *Campylobacter* from contaminated chicken products required enrichment for 18 h. Therefore, the effects of chemotactic stimuli on motility ability of viable *Campylobacter* to pass through a 0.45 µm pore size filter in viscous conditions were investigated. The system could permit more rapid detection of viable thermophilic *Campylobacter*. After 6 h, mucin-bile constituents at the concentrations of 1, 5, and 10% demonstrated significant increase in numbers of viable cells ($p < 0.05$). In addition, the role of starvation on chemotactic responses was also studied. Starved cells showed lower chemotactic response than non-starved cells significantly.

Detection of *Campylobacter* from 305 chicken samples was comparatively studied using both conventional method and the apparatus. The results revealed that the apparatus detected *Campylobacter* in 31 samples (10.16%) while 28 (9.18%) of these positive samples contained *C. coli* and 3 (0.98%) contained *C. jejuni*. However, the conventional method could only detect 14 (4.59%) samples positive with *C. coli*. In adition, *cdtB* gene was present in 100% (3/3) of *C. jejuni* isolated. Contrastly, this gene was not found in *C. coli* isolated. In conclusion, the apparatus detected more positive samples than did the conventional culture method.

Further study of the apparatus demonstrated that it was able to detect the presence of viable *C. concisus* and *Helicobacter pylori*, associated with human inflammatory bowel diseases, with a minimal bacterial density of 10^2 cfu/ml. Preliminary study with pure cultures was carried out for the isolation of *C. concisus* and *H. pylori* from gastric biopsies. In total, 5 biopsies were comparatively studied using both

conventional method and apparatus. Unfortunately, all samples were negative for *C. concisus* and *H. pylori*. However, a larger sample size is required for the validation of this application.

In summary, advantages of the apparatus are that it could detect only viable organisms. Detection time is within 24 h, 18 h required for isolation and 6 h for the identification using our developed multiplex PCR. The multiplex PCR system could detect the organisms from whole cells, therefore minimize time taken for DNA extraction. In addition, the system could detect *cdtB* gene that encodes CDT activity which is useful for epidemiological studies. The novel apparatus could offer a cheap, quick, and accurate system that would assure the quality of our products which would in turn have extremely strong economic impact.

CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

BAM	=	Bacteriological analytical manual
Bp	=	Base pair
cm	=	Centimeter
cfu	=	Colony forming unit
°C	=	Degree celcius
DNA	=	Deoxyribonucleic acid
FDA	=	Food and drug administration
g	=	Gram
h	=	Hour
l	=	Liter
pH	=	-Log hydrogen ion concentration
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
mg	=	Milligram
ml	=	Milliliter
mm	=	Millimeter
min	=	Minute
M	=	Molar
OD	=	Optical density
%	=	Percent
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
SD	=	Standard deviation
SE	=	Standard error of the mean
U	=	Unit

CHAPTER 1

INTRODUCTION

Campylobacter species are a major cause of bacterial diarrhea in humans worldwide and are a major concern to the poultry industry. It was first observed in 1886 but it was not until 1963 that the genus *Campylobacter* was established (Moore *et al*., 2005). In 1972, *Campylobacter* were recognized as causes of foodborne illness (Friedman *et al.,* 2000; Moore *et al*., 2005). *Campylobacter* are a Gram-negative, motile, spiral-shaped bacterium which exists as a commensal organism in the gastrointestinal tracts of a variety of wild and domestic animals (Friedman *et al.,* 2000). Currently, the genus *Campylobacter* includes 18 species (Humphrey *et al.,* 2007). In particular, *C. jejuni* is one of the most common causes of foodborne gastroenteritis.

Recently, foodborne outbreaks of *Campylobacter* disease appear to be increasing, especially in related to food production process, which can involve large numbers of infected subjects (WHO, 2007; CDC, 2008). Thermophilic *Campylobacter* especially *C. jejuni* and *C. coli* which can grow at 42°C, are most frequently isolated from faecal samples from diarrhoeal patients, particularly children (Friedman *et al.,* 2000). Furthermore, *C. jejuni* has been implicated as a frequent antecedent to the development of the neurologic disease, Guillan-Barre syndrome (Nachamkin, 2002). In addition, *C. lari* and *C. upsaliensis* have been infrequently isolated from humans, however, both have been reported to cause human infections (Goossens *et al.,* 1991; Soderstrom *et al.,* 1991; Martinot *et al.,* 2001; Werno *et al.,* 2002).

Poultry have been recognized as the primary reservoir of thermophilic *Campylobacter* and play an important role in transmission of *Campylobacter* to humans (Pearson *et al.,* 2000; Humphrey *et al.,* 2007). This finding has highlighted the need for microbiological quality control throughout the food chain production. For safety reasons, ready-to-eat products and poultry meat should be expected to be free of detectable *Campylobacter* species.

Conventional detection of *Campylobacter* from naturally-contaminated samples such as food and clinical specimens in many cases requires at least 4 days to grow due to the slow growth rate. Enrichment procedures are also required for *Campylobacter* (Friedman *et al.,* 2000). Moreover, identification of *Campylobacter* to species level requires additional times, resulting in up to 5 days being necessary to obtain a result (Corry *et al.,* 1995). Differentiation of *C. jejuni* and *C. coli* is currently made based on hippurate hydrolysis and antimicrobial sensitivity testing (cephalothin and nalidixic acid susceptibility). However, errors can occur in identification as some hippurate hydrolysis-negative *C. jejuni* (Steinhauserova *et al.,* 2001; Kos *et al.,* 2006) and a number of nalidixic acid-resistant *C. jejuni* (Gaunt and Piddock, 1996; Aarestrup *et al.,* 1997) have been reported. The isolation methodology currently employed in many diagnostic laboratories has been established for *C. jejuni* and *C. coli*, and theses may not facilitate the growth of other potentially pathogenic *Campylobacter* including *C. lari* and *C. upsaliensis*. Thus, the incidence of enteritis due to *C. lari* and *C. upsaliensis* may be underestimated. Many attempts have been made to develop more effective detection methods. A number of studies have examined to use of molecular techniques to detect thermophilic *Campylobacter*. Polymerase chain reaction (PCR) is a rapid specific nucleic acid amplification method for the detection of foodborne pathogens.

Major advantages of PCR-based detection method are their rapidity, specificity, and sensitivity. Currently, a number of PCR-based methods have been reported for the detection of *C. jejuni* and *C. coli* in poultry production (Giesendorf *et al.,* 1992; Oyofo *et al.,* 1997; Denis *et al.,* 1999; Grennan *et al.,* 2001), water (Sails *et al.,* 2002; Moreno *et al.,* 2003), and clinical samples (Linton *et al.,* 1997; Maher *et al.,* 2003). Multiplex PCR-based methods have been developed for the detection of thermophilic *Campylobacter* including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* in both clinical and environmental samples. However, large numbers of organisms are required to allow detection (Wang *et al.,* 2002; Klena *et al.,* 2004). A number of adaptations of these PCR assays have been made to improve their sensitivity including dot blot hybridization (Gonzalez *et al.,* 1997; van Doorn *et al.,* 1997), PCR enzyme-linked immunosorbent assays (Lawson *et al.,* 1999; O'Sullivan *et al.,* 2000), and real-time PCR (Nogva *et al.,* 2000; Sails *et al.,* 2003; Lund *et al.,* 2004). However, these methods are very expensive and therefore not a feasible option for use in developing countries. In addition, none of these methods can establish the viability of the organism.

An apparatus which can be used for the detection of viable thermophilic *Campylobacter* including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* has been developed. This apparatus is based on the motility observed in these *Campylobacter*. In addition, I have established a multiplex PCR for the identification of these four species and have compared this with conventional cultural methods. Furthermore, different DNA template preparation methods were investigated so that a high detection rate of *Campylobacter* could be achieved.

OBJECTIVES OF THE RESEARCH

1. To establish a novel apparatus which can be used under an aerobic condition for the detection of viable thermophilic *Campylobacter* in chicken meat products.
2. To establish a multiplex PCR for the identification of thermophilic *Campylobacter* and to assess the specificity of this PCR.
3. To compare this new method with conventional cultural methods.
4. To enhance the detection of thermophilic *Campylobacter* by chemotactic stimuli.
5. To determine the prevalence of *Campylobacter* species using this new culture method and using the multiplex PCR identify the species of *Campylobacter* in chicken meat.

CHEPTER 2

LITERATURE REVIEW

2.1 General characteristics

The bacteria now classified as *Campylobacter* were originally part of the genus *Vibrio*, however, they were separated into their own genus in 1963 (Friedman *et al.*, 2000). Until 1938, what are now termed *Campylobacter* were considered veterinary pathogens. In this year, the first human clinical report of two milk-borne outbreaks of enteritis was published. In 1968, a major breakthrough occurred when *Campylobacter* were isolated from human faeces. Currently, there are 18 species, six sub-species, and two biovars assigned to the genus *Campylobacter* (Nachamkin *et al.*, 2000; Humphrey *et al.*, 2007).

Campylobacter are Gram-negative, nonsporeforming, spirally curved rod shaped bacteria, 0.2-0.8 μm wide and 0.5-5 μm long. Most species within the *Campylobacter* genus are motile with a single unsheathed flagellum at one or both poles (Nachamkin *et al.*, 2003; Vandamme, 2000), which gives them a very characteristic 'corkscrew' motility. *Campylobacter* do not ferment carbohydrates and usually obtain energy from amino acids or tricarboxylic acid cycle intermediates (Nachamkin *et al.*, 2003). *Campylobacter* are microaerophilic, surviving optimally in atmospheres with 5-7% oxygen and approximately 10% carbon dioxide (Doyle and Jones, 1992; Corry *et al.*, 1995).

Pathogenic species in humans that have a temperature range for growth of between 30-46°C are classified as thermophilic *Campylobacter* (Humphrey *et al.*, 2007). Thermophilic *Campylobacter* do not grow at temperatures of less than 30°C and their optimal growth temperature is 42°C (Park, 2002). Thermophilic *Campylobacter* commonly associated with human gastroenteritis including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*.

2.2 Clinical features

Campylobacteriosis is the name given to the illness caused by *Campylobacter* infection. It is also known as *Campylobacter* enteritis or *Campylobacter* gastroenteritis and the infective dose has been shown to be as low as 500-800 cells (Black *et al.*, 1988). *C. jejuni* and *C. coli* are considered the most important *Campylobacter* for public health. Based on selective media culture in developed countries, *C. jejuni* and *C. coli* account for approximately 80-94% and 2-15% of human infections, respectively (Friedman *et al.*, 2000, Moore *et al*., 2005). Species other than *C. jejuni* and *C. coli* such as *C. upsaliensis*, and *C. lari* have been recently reported to be associated with human illness (Lastovica and Skirrow, 2000; Vandenberg *et al.*, 2006).

The infection has an incubation period of 7-10 days with symptoms being exhibited by 4 days (Pebody *et al.*, 1997; Allos, 2001). Most typically, infection with *C. jejuni* results in an acute illness characterized by diarrhea, fever, abdominal cramps, malaise, nausea, and vomiting. Long-term infection with *Campylobacter* has been linked to neurological and rheumatological diseases. Serious autoimmune disorders related to *C. jejuni* infection include Guillain-Barre syndrome (GBS), its variant Miller Fisher syndrome (MFS), and reactive arthritis (Nachamkin, 2002).

GBS is a polio-like form of paralysis that can result in respiratory symptoms and severe neurological dysfunction (Allos, 1997; Altekruse *et al.*, 1999; Nachamkin, 2002). It is estimated that 1 case of GBS occurs for every 1,000 cases of campylobacteriosis. MFS has also been associated with *C. jejuni* infection in humans. This syndrome is characterized by ophthalmoparesis, lack of reflexes, and incoordination without weakness (Nachamkin *et al.*, 2002). Cases of reactive arthritis post campylobacteriosis have also been reported with duration for complete recovery lasting from a few weeks to years (Nachamkin *et al.*, 2002).

2.3 Incidence of *Campylobacter* infections

Campylobacter cause bacterial gastroenteritis in people in both the developed and developing countries. In developed countries, *C. jejuni* have emerged as a significant public health problem. Data from World Health Organization (WHO) and the Centers of Disease Control and Prevention (CDC) have published that *C. jejuni* infections in some countries occur more frequently than infections caused by *Salmonella*, *Shigella*, or

Escherichia coli O157: H7 (WHO, 2007; CDC, 2008). In 1996, the Foodborne Diseases Active Surveillance Network (Foodnet) began the collection of data on 9 foodborne illnesses in the United States. From 1996-2000, the incidence rate of *C. jejuni* infected in selected states was approximately 20 per 100,000 population in the USA (CDC, 2000). In 2008, the *Campylobacter* incidence was reported as 12.68 per 100,000 population. Although a significant decline in the incidence of *C. jejuni* infections occurred between 1996 and 2004, the estimated incidence of *C. jejuni* infections have not changed a significantly in 2008 as compared with 2004-2007 (CDC, 2008). In this report, case fatality rates from *C. jejuni* infections were estimated to be approximately 0.4%. In most cases, the incidence was highest among children aged less than 4 years. In European countries, the number of reported cases of campylobacteriosis increased during the 1990s, with a total of 200,507 cases of campylobacteriosis in humans being reported in 2007 (EFSA, 2009). In non-European countries, the incidence was 120 cases per 100,000 inhabitants in Australia in 2007 (OzFoodNet, 2007).

In developing countries, the prevalence of *Campylobacter* in children in Southeast Asia ranges from 2.9-15% (Varavithya *et al.,* 1990; Taylor *et al.,* 1993; Phetsouvanh *et al.,* 1999; Bodhidatta *et al.,* 2007). In Southeast Asia, the incidence rate peak in *Campylobacter* infection has been reported in children less than 5 year old (Padungton and Kaneene, 2003). In Thailand, *Campylobacter* were the most common bacteria (28%) found in children in Bangkok, as compared with *Salmonella*, *Shigella*, and enterotoxigenic *Escherichia coli*. The isolation rates were 18%, 9% and 6%, respectively (Bodhidatta *et al.,* 2002). Moreover, in Thailand, between 1996-2004, the incidence of foodborne diseases increased from 137 to 248 cases per 100,000 population, then declined slightly to 217 cases per 100,000 people in 2006 (Ministry of Public Health, 2006). Most commonly-isolated pathogens from patients with foodborne illness in Thailand are rotavirus, *Salmonella* and *Campylobacter*.

2.4 Transmission

Campylobacteriosis is a zoonosis and an important public health problem in most areas of the world. Zoonotic infections are transmissible between animals and humans. *Campylobacter* can be transferred from animals to man directly after contact with animals or through consumption and handling of contaminated food products (Crushell *et al.,* 2004). *Campylobacter* are widespread in warm-blooded animals used for food

products, and consequently contaminate many meat products, and in particular poultry. Poultry are considered a primary source of *Campylobacter* infection to humans (Friedman *et al.*, 2000; Moore *et al*., 2005; Humphrey *et al.*, 2007). Poultry carcass contamination may vary from 10^2-10^5 *Campylobacter* cells per carcass (Jacobs-Reitsma and de Boer, 2001). In fact, by the fourth week of life chickens raised commercially are colonized with *C. jejuni* (Vandamme, 2000; Moore *et al*., 2005). One of the first documented outbreaks of campylobacteriosis, which was directly attributed to consumption of chicken, occurred in Netherlands (Brouwer *et al*., 1979). Several epidemiological studies have demonstrated a high prevalence of *Campylobacter* in poultry, ranging from 40-100% (Mateo *et al.*, 2005; Sallam, 2007; Katzav *et al.*, 2008; Williams *et al.*, 2008). Unpasteurized milk and contaminated water are also sources of *Campylobacter* infections (Said *et al*., 2003; Smith *et al*., 2006; CDC, 2007).

In developed countries such as North America and Europe, many studies have reported on the level of contamination with *Campylobacter* in retail poultry meat. For example, the prevalence of *Campylobacter* has been reported to be 79.0% in the USA (Nannapaneni *et al.*, 2005), 75% in Canada (Valerie *et al.*, 2009), 57-97% in the UK (Little *et al*., 2008; Moran *et al*., 2009), and 32-43% in Germany (Adam *et al.*, 2006) and 65-74% in the United States (Cui *et al*., 2005; Oyarzabal *et al*., 2007).

In developing countries, chicken products have been found to be important sources of *Campylobacter* infection in humans, both in Asia (Padungtod and Kaneene, 2005; Vindigni *et al.*, 2007) and Africa (Cardinale *et al.*, 2004; van Nierop *et al.*, 2004). In Thailand, *Campylobacter* were isolated from 15.5% of retail poultry meat (Vindigni *et al.*, 2007), as compared with 31% in Hanoi, Vietnam (Luu *et al*., 2006), 68-100% in Taipei, Taiwan (Shih, 2000), 77% in Nairobi, Kenya (Osano and Arimi, 1999), and 69.8% in Morogoro, Tanzania (Mdegela *et al.*, 2006). In Africa, *C. jejuni* was reported more frequently than *C. coli* in live chickens (Osano and Arimi, 1999; Cardinale *et al.*, 2004; van Nierop *et al.*, 2004). In northern Thailand, both *C. coli* and *C. jejuni* are highly prevalent in chickens, as well as in the chicken production system and chicken farms. On farms, *C. jejuni* has been reported to be the most prevalent (42.5%), followed by *C. coli* (39.1%). In contrast, *C. coli* has been reported to be the most prevalent *Campylobacter* at slaughter house and fresh chicken markets (72.4%), followed by *C. jejuni* (17.2%) (Meeyam *et al.*, 2004).

2.5 Pathogenesis

Several putative virulence factors have been identified in *C. jejuni*. The exact mechanisms by which *C. jejuni* induces disease in humans are still not completely understood, but the organism does produce a toxin and also is an invasive organism.

2.5.1 Flagella

In *C. jejuni*, motility is achieved by a single flagellum at one or both ends of the bacteria, and it has an important role in virulence because it is required for the bacteria to reach the attachment sites and penetrate into the intestinal cells. It is also reported that the flagella of *C. jejuni* appeared to have an essential role in the causation of diarrheal disease. The importance of motility as a virulence factor is best demonstrated by true isogenic non-flagellated mutants, which are unable to colonize the intestine of experimental animals (Guerry *et al*., 1992).

The role of flagella in the colonization of the mucous lining of the gastrointestinal tract has already been studied (Yao *et al*., 1994). Flagella are also important for invasion of host cells, as aflagellate organisms show markedly reduced internalization into host cells *in vitro* (Wassenaar and Blaser, 1999). The flagella of *C. jejuni* are composed of proteins, encoded by two genes *flaA* and *flaB* sharing a high degree of sequence homology (Wassenaar and Blaser, 1999). It has been shown that defined mutations in the *flaA* gene results in truncated flagella and diminished motility which are unable to invade intestinal epithelial cells *in vitro* (Wassenaar *et al*., 1991; Yao *et al*., 1994). Furthermore, a mutation in another gene encoding pyruvate formate lyase activating enzyme 1 (*pflA*) resulted in bacteria with paralyzed flagella that lead to a mutant which is still able to adhere, but is not capable of invasion *in vitro* (Yao *et al*., 1994). Therefore, it was proven that FlaA is not the only determinant that is critical for invasion of this pathogen *in vitro*.

The genome sequence analysis of *C. jejuni* strain 11168 led to the prediction of the involvement of more than 50 genes in the assembly of the flagella. The regulation of the *Campylobacter* FlaA-regulon seems to be more complex because the respective genes are located in more than 32 individual loci, while in *E. coli* they are located in only six loci. Despite the fact that the phenotypes of many non-motile mutants are known, the exact mechanism of regulation of the flagellar genes in *C. jejuni* is still not understood. Recently, some flagellar transcription activators like RpoN, FliA and FlgR

were identified, but how exactly these proteins are regulated and their role in the assembly of *C. jejuni* flagellum remains to be elucidated (Wösten *et al*., 2004).

2.5.2 Adhesion and invasion

There are several reports exploiting *in vitro* adherence assays to characterize the interaction of *C. jejuni* with host cells. It has been reported that C. *jejuni* is capable to bind to several cell lines of human (INT 407, HEp-2, and HeLa) and non-human origin (Vero, CHO-K1, and MDCK) with equal efficiency. Human intestinal epithelial (INT407) and human colon carcinoma cell lines (Caco-2) were thought to be good models to mimic those cells encountered by *C. jejuni in vivo* and therefore, these cell lines were extensively used to study adhesion of *C. jejuni.* The concept that adhesion of *C. jejuni* to host cells is mediated by constitutively synthesized products is supported by the finding that metabolically inactive *C. jejuni* organisms can bind to cultured cells at levels equal to or greater than those at which metabolically active, untreated *C. jejuni* cells bind.

A variety of putative adhesion factors of *C. jejuni* have been already identified which include the fibronectinbinding protein CadF (Konkel *et al*., 1997), a homologue of the gram-negative ABC transport system PEB1 (Pei and Blaser, 1993) and a major outer membrane protein (Moser *et al*., 1997). Recently, it has been suggested that a novel surface-exposed lipoprotein specific to *C. jejuni* plays a role in host cell adherence (Jin *et al*., 2001). The lack of a suitable and accessible animal model of infection is a major hindrance to determine the precise role of these potential virulence factors to human disease. A significant variation has been observed in the degree of invasiveness between different strains of *C. jejuni* (Newell *et al.*, 1985; Konkel and Joens, 1989).

Invasion of *C. jejuni* has been shown in colonic epithelial cells taken from infected humans and macaque monkeys (Woolridge and Ketley, 1997). Different experiments on a variety of cell lines including human intestine-derived Caco-2 and INT 407 cells showed invasiveness (Woolridge and Ketley, 1997; Kopecko *et al*., 2001). Some isolates of *C. jejuni* such as the well characterized strain 81176 are proven to be highly invasive in these experimental models, however, many other isolates show low levels of host cell entry *in vitro* (Kopecko *et al*., 2001). Recently, entry of *C. jejuni* in polarized epithelial cells via the basolateral membrane has been demonstrated as well as there is evidence for paracellular passage and M-cell transcytosis (Woolridge and Ketley, 1997). Some studies demonstrated microtubule-dependent invasion of *C. jejuni* 81176 and its

reliance on microtubule motors for uptake and intracellular motility (Hu and Kopecko, 1999; Bourke, 2002). Likewise, most strains of *C. jejuni* showed microfilament or microtubule-dependent invasiveness (Biswas *et al*., 2000).

2.5.3 Toxin production

C. jejuni-induced clinical symptoms, which often include a transient watery diarrhea that progress to a bloody diarrhea, are consistent with the idea that toxins play a role in this disease. Indeed, a variety of toxic activities has been reported in *C. jejuni*. However, cytolethal distending toxin (CDT) is the only verified *Campylobacter* toxin identified to date. CDT production by *Campylobacter* was first reported in 1988 (Johnson and lior, 1988). During the last few years, significant progress has been made to understand the cellular effect of CDT (Hickey *et al*., 1999; Karlyshev *et al*., 2001). In 1996, the isolation and characterization of the *cdt* genes from *C. jejuni* 81176 was reported and it is now well known that CdtB is the active moiety of the Cdt ABC complex (Pickett *et al*., 1996). It appears that CdtA and CdtC interact with CdtB to form a tripartite CDT holotoxin necessary for the delivery of the enzymatically active subunit, CdtB (Lara-Tejero and Galan, 2001). It has also been shown by Whitehouse *et al*. (1998) that affected epithelial cells undergo cytodistension and cell cycle arrest in the G2/M phase. T lymphocytes exposed to CDT-mediating sonicates from *C. upsaliensis* showed cell cycle arrest. The role of CDT in *C. jejuni* pathogenesis has not been determined yet, however, it might play a role in modulation of immune response and invasiveness (Purdy *et al*., 2000).

Another increasingly recognized prokaryotic virulence mechanism is to subvert host cell processes by targeting bacterial products directly to the cytoplasm of the host. Konkel *et al*. (1999) reported a *C. jejuni* protein called CiaB that seems to enter host cells during the invasion process. It has been shown that isogenic CiaB mutants were deficient in secretion of a number of bacterial proteins. A flagellar export system encoded by the genome of *C. jejuni* 11168 has been reported, but there is no evidence for the presence of a typical type III secretory apparatus. Recently, Bacon *et al*. (2002) identified homologues of a type IV secretory apparatus on a large plasmid of *C. jejuni* 81176. The 37kb the pVir plasmid of *C. jejuni* harbours 54 predicted open reading frames. In some studies, it has been shown that mutations in some of the plasmid-encoded genes might reduce invasion compared with the parental strain *in vitro*. However, transfer of the

plasmid to the sequenced strain, NCTC 11168, did not show any difference in the invasiveness of this isolate (Bacon *et al*., 2000).

2.6 Chemotaxis

Chemotaxis defined as the movement of an organism towards or away from a chemical stimulus. Chemotaxis has been noted to be an important factor in the colonization of pathogenic bacteria including *V. cholerae, S. typhimurium*, and *E. coli.* Several studies demonstrated that chemotaxis is an important virulence determinant in *C. jejuni* and plays an important role in the colonization of mice. The *cheY* null mutant of *C. jejuni* was generated (Yao *et al*., 1997) and found to display a nonchemotactic but motile phenotype. A three-fold increase in the adherence and invasion of INT 407 cells was noticed as compared to the wild type with the *che*Y null mutant, which was unable to colonize mice or cause symptoms in infected ferrets. In the same study, it was shown that *cheY* diploid isolates showed a chemotactic behaviour and a decrease in their *in vitro* adherence and invasion capabilities. Although, this isolate was able to colonize mice, it was unable to cause disease in the ferret model. It was suggested that these bacteria migrated towards the mucus within the crypts, but were unable to penetrate the mucus (Konkel *et al*., 2004).

2.7 Antimicrobial resistance

Antimicrobial resistance is a major concern to Public Health Authorities which has led to the initiation of the National Antimicrobial Resistance Monitoring System (NARMS) in USA. This system works collaboratively with the Food and Drug Administration's Center for Veterinary Medicine, United States Department of Agriculture (USDA) and CDC to monitor antimicrobial resistance including human nontyphoidal *Salmonella, E. coli* O157 and *Campylobacter* (FSIS USDA, 2001; NARMS US FDA, 2007). Due to unrestrict regulation on the use of antibiotics in most developing countries, the prevalence of macrolide and fluoroquinolone-resistant *Campylobacter* is rapidly increasing.

In Thailand, ciprofloxacin resistance rates of *Campylobacter* have increased from 0% to 84% between 1991 and 1995 (Hoge *et al.*, 1998). Ciprofloxacin resistance was reported to be 6% among Thai patients, but 76% among Swedish travellers

(Gibreel *et al.,* 1998). It has been suggested that fluoroquinolone resistance may occur following treatment with ciprofloxacin as empirical therapy for gastroenteritis patients with traveller's diarrhea including those associated with *Campylobacter* infection (Zirnstein *et al.,* 1999; Allos, 2001). Moreover, poultry and farm animals may act as reservoirs for as well as a significant source of fluoroquinolone-resistant *Campylobacter* (Aarestrup and Wegener, 1999; Minihan *et al.,* 2006). Fluoroquinolones act by inhibiting the activity of DNA gyrase (Oliphant and Green, 2002; Hawkey, 2003) and DNA topoisomerase IV (Hooper, 2000) which are involved in bacterial DNA synthesis. The enzyme DNA gyrase introduces negative supercoils in the bacterial DNA double helix ahead of the replication fork, thereby catalyzing the separation of daughter chromosomes. The role of topoisomerase IV appears to be associated with decatenating the daughter replicons. Both enzymes are encoded by *gyrA/gyrB* and *parC/parE,* respectively (Hawkey, 2003).

There are three main mechanisms of quinolone resistance including (i) alteration of the quinolone target enzymes, (ii) reduction of the accumulation of the drug in the cell via efflux pump systems (Charvalos *et al.,* 1995; Lin *et al.,* 2002; Piddock *et al.,* 2003; Randall *et al.,* 2003), and (iii) transferability quinolone resistance via plasmid carrying quinolone resistance (*qnr*) gene (Tran and Jacoby, 2002; Jacoby *et al.,* 2003). Quinolone resistance mechanisms mediated by target changes have been found in *gyrA*, *gyrB*, *parC*, and *parE* genes. The hot spot for mutations has been shown to be in the quinolone resistance determining region (QRDR) which is specific for quinolone binding. Thr-86-Ile is the predominant point mutation in *gyrA* mostly found in association with quinolone resistance and increase in the minimum inhibitory concentrations (Zirnstein *et al.,* 1999). *Campylobacter* multidrug efflux, CmeABC, a member of the resistance nodulation division (RND) family, has been described in *C. coli* and *C. jejuni* which contributes to the intrinsic and acquired resistance of multidrugs and agents (Corcoran *et al.,* 2005; Lin *et al.,* 2005).

2.8 Viable but non-culturable organism

In the environment, microorganism face a series of stressful conditions, such as nutrient starvation, osmotic shock, and temperature variation, forcing them to activate survival strategies which include entering the viable but non-culturable (VBNC) (Rollins and Colwell, 1986; Korhonen and Martikainen, 1991). These microorganisms, although expressing various degrees of metabolic activity and possibly being able to cause

infections, cannot be cultured on conventional laboratory media which would normally support their growth (Oliver, 2005). In this state, microbial cells lose their viability very quickly as measured by plate count on agar (Rollins and Colwell, 1986). The VBNC state has been reported in *C. jejuni* (Rollins and Colwell, 1986; Stern, 1994; Hazeleger *et al.*, 1995). Resuscitation from the VBNC state is one of the major concerns. Some authors have reported *C. jejuni* resuscitation from the VBNC state after passage in experimental animals (Jones *et al.*, 1991; Cappelier *et al.*, 1999). In addition, changes in the morphological structure of *Campylobacter* from spiral to coccoid have been observed during environmental stress as well as in old cultures (Ng *et al.*, 1985). The coccoid form tends to be difficult to subculture and have been shown to lose motility (Oliver, 2005).

However conflicting evidence as to the importance of coccoid forms, injured cells and the VBNC forms exists, with strain-to-strain variation suggested as an explanation (Jones *et al.*, 1991). Some VBNC strains do not display coccoid morphology and were not able to colonize birds (Fearnley *et al.*, 1994), while other injured cells have been found to resuscitate after passage through animal and protozoal hosts (Saha *et al.*, 1991; Axelsson-Olsson *et al.*, 2005). Further, injured strains have also been culturable but not able to colonize avian hosts and have been referred to as culturable but not infectious (Hald *et al.*, 2001). The ability of injured strains to be non-culturable and yet revert to virulence with passage through an appropriate host may be epidemiologically important for identification of transmission routes to people.

2.9 Food contamination and infection

Although generally, *Campylobacter* colonize in high concentrations in the cecum and colon of poultry, they can also be found in the crop (Friedman *et al*., 2000). Since thermophilic *Campylobacter* grow optimally at temperatures near 42°C, the higher metabolic temperatures present in poultry may predispose them to be the prominant reservoir for thermophilic *Campylobacter* (Park, 2002).

Normal food storage (4°C) conditions do not allow the growth of *C. jejuni* because of its relatively high temperature requirements for growth. However, *C. jejuni* can survive on chicken during processing and storage of food. Indeed, it has been shown that *C. jejuni* can survive for extended periods in refrigerated meat (Gill and Harris, 1982; Chan *et al.*, 2001). The survival times for *C. jejuni* vary widely from 2-4 weeks to as long as 4 months at low temperatures, particularly between 4°C and 10°C (Thomas *et al.*, 2002;

Cools *et al.*, 2003). Due to the ability of these *Campylobacter* to survive in food at low temperatures. In many countries routine surveillance of microorganisms in food increasingly includes thermophilic *Campylobacter*.

In Thailand, chickens are the major exported food commodities. The Department of Livestock Development (DLD) have approximated that at any given time on Thai farms, there are 250 million chickens (DLD, 2007). Thailand is the largest chicken-exporting nation in Asia, with Japan being the largest markets of Thai export chicken. The United States Department of Agriculture Foreign Agricultural Service (USDA FAS) has reported that Thailand's production of chicken exports totaled 401,474 tons. These exports earned Thailand 55,423 million Baht in 2008 (FAS, 2008). Under the Japan-Thailand Economic Partnership Agreement, Thailand's agricultural exports stand to reap considerable benefit from Japan's import duty reductions as well as the opening up of its market to Thai products. Japan and the European Union (EU) imported 46.11% and 44.65% of the total cooked chicken, respectively. The remaining 4% of Thai chicken exports were frozen chicken and the markets included Japan, Vietnam, Singapore, South Korea, and Hong Kong and the EU. According to the Thai Broiler Processing Exporters Association in the first three months of 2009, Japan and EU remained major markets for Thai chicken meat exports, accounting for 47% and 44%, respectively. Other importing countries include Vietnam, Singapore, South Korea, and Hong Kong. Livestock farming in Thailand, particularly on chicken farms, has been inspected by the DLD and certified to meet the standards of practice required by law. However, there are no specific criteria for *Campylobacter* (DLD, 2008).

Campylobacter are the leading causative agents of foodborne bacterial illnesses in Japan. In more than 40% of the cases in which the cause was identified, the consumption of poultry meat or by-products was suspected as the cause, indicating a strong relationship between campylobacteriosis and the consumption of poultry (The Ministry of Health, Labour and Welfare, 2007). In Japan, domestic poultry included both poultry meat and by-products, approximately 90% of isolates were shown to be *C. jejuni*, while approximately 10% were *C. coli*. In contrast, it was observed that *C. coli* was isolated more frequently from imported poultry than from domestic poultry (The Ministry of Health, Labour and Welfare, 2007). Approximately 20-40% of poultry meat and by-products were found to be contaminated with *Campylobacter* at the level of 1,000 cells per 100 g (Suzuki and Yamamoto, 2009). In addition, frozen poultry imported from Thailand showed a relatively high prevalence of *Campylobacter* contamination (Suzuki and

Yamamoto, 2009). Currently, there is no official protocol in Japan for detecting the presence of *Campylobacter* species in food stuffs.

In 2007, the annual community summary report by the European Food Safety Authority and the European Centre for Disease Prevention and Control reported campylobacteriosis to be the most commonly reported zoonotic disease in humans in the EU, with 200,507 confirmed cases reported which represented 12% overall increase compared to 2006 (Westrell *et al*., 2009). In foodstuffs, *Campylobacter* was most commonly detected in fresh broiler poultry meat where on average 26.0% of samples were found positive. *Campylobacter* were also frequently found in animals and most often in poultry flocks and pigs. In the broiler flocks tested in the EU, 25.2% were positive for *Campylobacter* (Westrell *et al*., 2009).

In order to prevent and control zoonoses, it is important to identify which animals and foodstuffs are the main sources of infections. For this purpose and to monitor the progress on food safety in the EU, information aimed at protecting human health is collected and analysed from all EU member states according to the zoonoses directive 2003/99/EC (The European Parliament and the Council of the European Union, 2003). Member states must ensure that the monitoring system provides relevant information at least with regard to a representative number of isolates of *Salmonella*, *C. jejuni* and *C. coli* from cattle, pigs and poultry and food of animal origin derived from those species.

2.10 Methods for detection and identification of thermophilic *Campylobacter* from foods

Although there is no generally accepted ‘standard’ method of isolating *Campylobacter* from food, protocols have been published by recognized authorities including the International Standards Organisation (ISO) and the US Food and Drug Administration (US FDA). US FDA, (2001) and ISO, (2006) standard method are consisted of four steps including sampling, broth enrichment, isolation on selective plating media and phenotypic identification.

2.10.1 Cultural-based methods

In food samples, where cell numbers can be low in a background of high numbers of other competing microbiota, enrichment culture in broth media is required to recover small numbers of cells prior to plating on selective media (Corry *et al*., 1995).

2.10.1.1 Media

The development of Skirrow medium was the key to successful study of thermophilic *Campylobacter*. This medium enabled successful recovery of *C. jejuni* and *C. coli* (Skirrow, 1977). Although Skirrow's medium was effective for isolating *Campylobacter* undertaken from human faeces, it was less suitable for animal and environmental specimens (Corry *et al.,* 1995). Owing to the presence of contaminating species, the more selective Preston medium for isolating *C. jejuni* and *C. coli* from foods and environmental samples was developed by Bolton and Robertson (1982). However, even with these improvements, cultural-based methods face a number of limitations. Isolation can take several days to a week, selective media for *Campylobacter* are expensive, and identifying this bacterial species may be difficult (Corry *et al.,* 1995).

All *Campylobacter* media contain peptones and antibiotics, most contain blood, and as well, many include oxygen quenching agents to overcome the adverse effects of toxic oxygen species. These ingredients form the basis of most *Campylobacter* media in common use. Preston broth (Bolton and Robertson, 1982) and Exeter broth (Martin *et al.*, 1996) contain meat extract and peptone. Park and Sanders broth (Park and Sanders, 1991) compose of peptones and yeast extract. Bolton broth and *Campylobacter* enrichment broth (CEB) have a nutrient formulation that consists of peptones, yeast extract and a tricarboxylic cycle intermediate, α-ketoglutaric acid (Bolton *et al*., 1984).

2.10.1.2 Antibiotics

Addition of antibiotics to isolation media is crucial for the recovery of *Campylobacter*. Since the organisms have been reported to be resistant to several antibiotics, these drugs could be used in order to select growth of *Campylobacter* including vancomycin (inhibits Gram-positive cocci), polymyxin B (inhibits *Enterobacteriaceae* and *Pseudomonas*), Trimethoprim (inhibits *Proteus* and Gram-positive cocci), and

cephalosporins (inhibit *Enterobacter, Serratia, Pseudomonas aeruginosa, Proteus, Yersinia enterocolitica*). Rifampicin was substituted for vancomycin in Preston media (Bolton and Robertson, 1982), but later studies demonstrated that rifampicin produced inhibitory effect to stressed *C. jejuni* cells (Humphrey and Cruikshank, 1985; Humphrey, 1990). Antibiotics which inhibit yeasts and moulds are usually included in *Campylobacter* media. Cycloheximide was the most widely used antifungal antibiotic, however, it is considered toxic for inclusion in microbiological media. Amphotericin B has been demonstrated to be a satisfactory substitute for cycloheximide (Martin *et al.*, 2002).

It is to be note that a number of antibiotics that are commonly used in *Campylobacter* media can adversely affect recovery of some species or strains. For example, Nachamkin *et al.* (2000) reported that cephalothin, colistin and polymyxin B may inhibit some strains of *C. jejuni* and *C. coli, C. upsaliensis,* and also *C. fetus*. Delayed addition of antibiotics may enhance recovery of thermophilic *Campylobacter*. For example, in some protocols inoculated broths are incubated for 4 h at 37°C, followed by addition of antibiotics and then transfer to a microaerophilic atmosphere at 42°C for 44 h (Wallace, 1997; Jones *et al.*, 1999). It is likely that future developments in *Campylobacter* media will be based on changes to optimize recovery of species other than *C. jejuni* such as supplement cefoperazone, amphotericin, and teicoplanin have been applied for selective isolation of *C. upsaliensis* (Oxoid Manual, 1998).

2.10.1.3 Reducing agents

Many *Campylobacter* media contain blood at levels 5-15% to quench toxic oxygen compounds (Bolton *et al.*, 1984). Some media use defibrinated or lysed blood from various animals. In addition, blood neutralizes trimethoprim antagonists (Corry *et al.*, 1995). Many media which incorporate trimethoprim do not contain lysed horse blood (Corry *et al*., 1995).

A combination of ferrous sulphate, sodium metabisulphite, and sodium pyruvate (FBP) was suggested by George *et al*. (1978) as an addition to *Campylobacter* media to counteract the toxic effect of oxygen. FBP have been shown to enhance the growth of *Campylobacter* in both supplemented agar and broth (George *et al.,* 1978). Many selective media contain some or all of these compounds but concentrations vary. A few media contain both FBP supplement and blood (Stern *et al.*, 1992).

Alternatives to blood or FBP include haematin and charcoal. The mode of action of these supplements is not clear, but they may help to neutralize hydrogen peroxide, singlet oxygen, and superoxide ions. Incorporation of charcoal to the medium can be used as an alternative of blood and it has been reported that charcoal could effectively replace the blood in the media formulations (Bolton *et al*., 1984).

2.10.1.4 Microaerobic conditions

Many approaches to obtain the microaerobic atmosphere required for the growth of *Campylobacter* have been reported. Several manufacturers produce microaerobic gas generator packs that are convenient for routine use. Evacuation of an anaerobic incubator and replacement with the appropriate gas mixture has been used for routine cultures (Morris and Patton, 1985). Adler and Crow (1981) first used membrane fractions extracted from *E. coli* in bacteriological media in order to achieve anaerobiosis. Oxygen-reducing membrane fragments have been used to provide microaerobic condition suitable for isolation of *Campylobacter* from food samples including chicken carcasses, meats, and milk (Raben and Slavik, 1994; Abeyta *et al.,* 1997; Wonglumsom *et al.,* 2001). Currently, microaerobic conditions are usually created by the gas jar system using either gas-generating envelopes or an evacuation-replacement procedure, the shaking gassed system when a flushing gas mixture consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen is introduced into each flask or bag manually, or by evacuating and replacing with the gas mixture, and bubbler system using continuous flow of mixed gas through the culture (US FDA, 2001). These common methods for generating microaerobic condition for growth of *Campylobacter* are labourious, expensive, time consuming or troublesome, and require special equipment.

2.10.1.5 Enrichment

Food samples are usually contaminated with relatively low numbers of *Campylobacter*. The incorporation of enrichment procedures into laboratory protocols has been found to increase recovery of *Campylobacter* from most sample types and is generally recommended for analysis of food, water and other environmental samples (Bolton *et al.*, 1984). Enrichment usually starts with a resuscitation procedure that is included to

overcome damage to cells caused by drying, heating, starving, freezing, and oxygen radicals. Probably the most widely used resuscitation procedure consists of 4 h incubation at 37°C after which the pre-enrichment broths are transferred to 42°C (US FDA, 2001; ISO, 2006). It is recommended that resuscitation be limited to 4 h to prevent overgrowth by contaminants (Goosens and Bultzer, 1992). However, Humphrey (1989) reported optimal recovery of *C. jejuni* from river water by selective isolation in broth for 48 h at 37°C. Following enrichment, an aliquot of growth from enrichment tubes is subcultured to a chosen selective agar that is usually incubated for 24-48 h at 42°C to confirm the presence of thermophilic *Campylobacter* (US FDA, 2001; ISO, 2006).

Bolton broth is recommended in protocols produced by the US FDA and ISO for isolation of *Campylobacter* from foods (US FDA, 2001; ISO, 2006). Enzymatic digest of animal tissues, lactalbumin hydrolysates, and yeast extract provide essential growth nutrients like vitamin, amino acids and other nitrogenous compounds to *Campylobacter* (Bolton, 1995). The additions of sodium metabisulphite and sodium pyruvate quench toxic compounds and increase on this way the recovery rate and also the aero-tolerance of the culture (Post, 1995). The α-ketoglutaric acid is used for an initial burst of the metabolism. Sodium carbonate is added to neutralize the acid that may form in the culture medium. The osmotic balance is given by the sodium chloride.

The antibiotics including vancomycin, cefopeerazone, and trimethoprim present in the supplement inhibit the growth of Gram-positive and Gram-negative bacteria. Amphotericin B, as well in the supplement, largely reduces the growth of yeasts and moulds (Bolton, 1995). Also the incubation temperature of 42°C, after the initial incubation step, increases the selectivity (Oxoid Manual, 1998). In the protocol, a microaerophilic atmosphere is specified for incubation, achieved using either using commercial *Campylobacter* gas paks, or an incubator gassed with a flowing mixture of 5% oxygen, 10% carbon dioxide and 85% nitrogen. For food sample, the enrichment period consists of incubation for 4 h at 37°C. Following resuscitation, enrichment broths are transferred to 42°C (US FDA, 2001; ISO, 2006).

2.10.1.6 Selective plating media

Campylobacter selective agars include blood-containing and charcoal-containing media. Skirrow, Preston, and Exeter media include blood. Blood-free selective

agars can include charcoal as an oxygen quencher, for example, Karmali or modified charcoal cefoperazone deoxycholate agar (mCCDA). mCCDA is recommended as a good selective agar by both US FDA and ISO (US FDA, 2001; ISO, 2006). mCCDA is based on the original formulation described by Bolton *et al*. (1987) which was developed to replace blood with charcoal, ferrous sulphate, and sodium pyruvate. Improved selectivity was achieved when cephazolin in the original formulation was replaced by cefoperazone as the selective agent. Amphotericin B has been added to the formula to suppress the growth of yeast and fungal contaminants that may occur at 37°C. Following enrichment, the isolation procedure continues with sub-culture to *Campylobacter* selective agar plates. A 10 µl loopful of enrichment was streaked to single colonies on mCCDA and incubated for 24-48 h at 42°C (US FDA, 2001; ISO, 2006).

Baylis *et al*. (2000) compared the performance of enrichment broth for the recovery of *Campylobacter* from food using both artificially and naturally contaminated samples. Popular enrichment broth include Bolton broth, CEB, and Preston broth. A variety of poultry and meat samples were artificially contaminated with reference cultures to determine quantitative recovery of *Campylobacter*. All enrichments included an initial period of resuscitation, 4 h at 37°C, after pre-enrichment all were subcultured to mCCDA agar. The results showed that Bolton broth and Preston broth supported growth of the greatest numbers of *Campylobacter* strains. However, Preston broth failed to inhibit some competitor organisms. In contrast, CEB inhibited all competitors but failed to support all of the *Campylobacter* strains. When compared recovery from naturally contaminated samples, Bolton broth detected more positive samples than did Preston or CEB broth.

2.10.1.7 Phenotypic identification

Typical smooth, convex, translucent, colorless to cream-colored colonies on mCCDA plates and deep red to magenta, smooth, shiny, and convex colonies with a defined edge or flat colonies with an irregular edge on blood containing plates (Nachamkin, 2003). Confirmation of *Campylobacter* is based on colony morphology, microscopic appearance and the phenotypic characteristics including production of oxidase and catalase, and hippurate hydrolysis reaction as described by US FDA (2001) (**Table 1**). This may lengthen the detection process with up to 5 days being required to achieve a result (Corry *et al*., 1995).

Table 1. Phenotypic identification of thermophilic *Campylobacter* species.

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
Growth at 25°C	-	-	-	-
Growth at 37°C	+	+	+	+
Growth at 42°C	+	+	+	+
Nitrate reduction	+	+	+	+
Catalase	+	+	+	-
Oxidase	+	+	+	+
MacConkey's agar	+	+	+	-
Glucose utilization	-	-	-	-
Hippurate hydrolysis	+	-	-	-
Naladixic acid	S	S	R	S
Cephalothin	R	R	R	S

+, 90% or more of strains are positive. -, 90% or more of strains are negative.

R, resistant. S, susceptible.

Furthermore, the conventional tests for the discrimination of *C. jejuni* and *C. coli* are cumbersome and subjective since these tests differentiate on the basis of a single phenotypic characteristic, the ability to hydrolyze hippurate. While *C. jejuni* generally give a positive reaction, about 10% of *C. jejuni* isolates fail to hydrolyse hippurate under laboratory conditions (Steinhauserova *et al*., 2001; Englen *et al*., 2003). Therefore, there are other methods available for the identification of *Campylobacter* including antibody-base methods and molecular-based methods.

2.11 Antibody-based methods

The most widely accepted method that uses a passive hemagglutination technique to detect heat-stable antigens was developed by Penner and Hennessy (1980). The variability of the *Campylobacter* lipopolysaccharide outer core and O polysaccharide is thought to contribute to the antigenic basis of the Penner serotyping system. The other scheme developed by Lior *et al*. (1982) is a slide agglutination test based on heat-labile antigenic factors. However, phenotypes can be unstable, resulting in non reproducible results or high numbers of untypeable strains (Penner and Hennessy, 1980; Lior *et al.,* 1982).

The development of both monoclonal and polyclonal antibodies specific for *Campylobacter* has facilitated the development of a number of antibody-based methods. Latex agglutination tests for culture confirmation have been developed (Nachamkin and Barbagallo, 1990). An immunomagnetic separation (IMS) hybridization assay for capturing and detecting *Campylobacter* cells from chicken meat has also been described (Lamoureux *et al.,* 1997). The detection limit of the IMS rRNA probe-rDNA hybridization system for cells in pure culture was reported to be 10^4 cfu/ml and the detection limit of the more specific IMS DNA probe-RNA system was 10^8 cfu/ml. This assay provided more rapid results when compared with conventional culture, results being available within hours rather than days. A commercial enzyme-linked immunosorbent assay has also been developed for the detection of *C. jejuni* and *C. coli* directly in faecal samples (Tolcin *et al.,* 2000). This assay was demonstrated to have a sensitivity of 96% and a specificity of 99%. However, these techniques are technically demanding and antisera are expensive (Lukinmaa *et al.,* 2004).

2.12 Molecular-based methods

Molecular genotype-based methods represent an alternative method to the identification of *Campylobacter* by phenotype-based methods. A number of conventional polymerase chain reaction (PCR) assays have been described for the identification and characterization of *Campylobacter* from a spectrum of sample types including stools (On and Jordan, 2003) and food products (Bang *et al.,* 2002; Burnett *et al.,* 2002; Sails *et al.,* 2003). Several variations of the standard PCR, multiplex PCR, and real-time PCR have been developed and these have assisted in producing more sensitive detection methods.

The first application of PCR for the specific detection of *C. jejuni* and *C. coli* was described in 1992 (Oyofo *et al.,* 1992). This assay targeted the flagellin A gene present in *C. jejuni* and *C. coli* and successfully detected as few as 30-60 bacteria cells in human faecal samples per PCR reaction. The first report of a PCR assay for the detection of *Campylobacter* in food was reported by Giesendorf (Giesendorf *et al.,* 1992). This assay demonstrated a limit of detection of 25 cfu of *Campylobacter* per g of chicken tissue following the 18 h enrichment process. Moreover, Grennan *et al*. (2001) have also described a PCR-ELISA for the detection of *Campylobacter* and the discrimination of *C. jejuni* and *C. coli* in poultry samples. The PCR assay targeted the 16S/23S ribosomal RNA intergenic spacer region of *Campylobacter* with DNA oligonucleotide probes. The study showed that PCR-ELISA, when combined with culture pre-enrichment, was able to detect the presence of *Campylobacter* and definitively identify *C. jejuni* and *C. coli* in culture-enriched poultry meat samples.

Multiplex PCR allows several targets to be co-amplified in one PCR reaction by combining primer pairs. Nayak *et al*. (2005) developed a multiplex PCR assay to identify *C. jejuni* and *C. coli*, using oligonucleotide primers that encode for virulence factors. In their study, PCR amplification of the isolates identified a 160 bp oxidoreductase gene, specific for *C. jejuni*, a 400 bp *cadF* gene encoding adhesion proteins in *Campylobacter* species, and 894 bp *ceuE* gene encoding lipoproteins, specific for *C. coli*. The cross-reactivity of the assay with non-*Campylobacter* strains were determines and the specificity of this assay to detect *C. jejuni* or *C. coli* was reported to be 97%. Wang *et al*. (2002) have also described a multiplex PCR using six sets of primers that amplify regions of *hipO* and the 23S rRNA from *C. jejuni*, *glyA* from each of *C. coli*, *C. lari*, and *C. upsaliensis*, and *sapB2* from *C. fetus* subsp. *fetus* genes. By analysing 137 clinical and environmental isolates, this method was found to be a rapid and easy to perform with

a high level of sensitivity. The assay could successfully distinguish five species of *Campylobacter* from each other.

Conventional PCR detection needed to prevent cross contamination with amplified DNA. For this reason real-time PCR has been developed (Lund *et al.*, 2004). Sails *et al.* (2003) reported the first application of a quantitative PCR assay to detect *C. jejuni* in naturally contaminated food. The assay was as sensitive as conventional culture methods but required a 48 h selective enrichment. In this study, a 24 h enrichment period was enough to detect all positive samples, which would significantly reduce the total time required for the detection of *Campylobacter* in foods. Nogva *et al*. (2000) have reported a 5'-nuclease PCR assay for the quantitative detection of *C. jejuni*. This technique was reported to provide relatively good discrimination between exposed DNA from dead *C. jejuni* and protected DNA in living bacteria. Although, the method can also amplify DNA from dead cells, increasing the number of an organism by enrichment culture make the detection of living cells more sensitive (Keer and Birch, 2003).

DNA microarray technology involves binding specific gene sequences to solid substrate using complementary base pair hybridization. On spotted arrays, each spot on the substrate may represent thousands of replicates of a particular genetic sequence (Taboada *et al.*, 2007). Array hybridizations are usually quantified through the use of fluorescent labeling of DNA (Draghici, 2003). DNA microarray may be used for both whole genome or for specific gene expression analysis. Microarray technology may also be used for detection of *Campylobacter* in mixed microbial populations. You *et al.* (2008) demonstrated that by using microarray technique for the detection and identification of *C. jejuni*. The virulence gene including *mapA*, *ceuE*, and *cdt* was chosen as the amplification target. In addition, using 16S rDNA and 23S rDNA genes as the target sequences to develop a system based on oligonucleotide microarray and to detect *C. jejuni*. The sensitivity of the developed oligonucleotide microarray could reach 10^3 cfu/ml (Xing *et al.*, 2008).

DNA microarrays were also used for the detection of *Campylobacter* in food samples. An electronic oligonucleotide microarray technique was developed for detection and differentiation of the viable *Campylobacter* species, *C. jejuni*, *C. coli*, and *C. lari* in chicken samples. This is achieved by using mRNA of the 60 kDa heat-shock protein as the viability marker. This technique was able to detect as few as two viable *Campylobacter* cells (Zhang *et al.*, 2006). Array methods may be expensive, with lack of protocol standardization between laboratories.

There has recently been an interest in the use of RNA as the molecular target for viability assessments of *Campylobacter*. Both messenger RNA (mRNA) and ribosomal RN (rRNA) should provide a better indication of viable organisms rather than DNA. In addition, rRNA has been found to be positively correlated with viability under some bacterial-killing regimes (McKillip *et al.,* 1998; Meijer *et al.,* 2000). That makes rRNA less accurate indicator of viability than mRNA targets. However, if mRNA is used, it is important to ensure that the mRNA targeted for transcription is present under most assay conditions (Keer and Birch, 2003).

The most commonly used amplification techniques for detecting RNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence based amplification (NASBA). RT-PCR is a two-stage process in which a target RNA sequence is first transcribed into a complementary DNA that then serves as the template for PCR (Chan and Fox, 1999). In contrast, NASBA is a one-step process in which single-stranded RNA sequences are targeted and amplified (Simpkins *et al.,* 2000).

Investigators have applied the technique of RT-PCR to detect viable thermophilic *Campylobacter* based on the detection of mRNA (Sails *et al.,* 1998). The assay can differentiate between viable and dead cells of *C. jejuni*. However, Sung *et al*. (2005) indicate that mRNA from *Campylobacter* may persist in a form that is detectable by RT-PCR amplification for an extended period after heat treatment, demonstrating a poor correlation between mRNA detection and cell cultivability.

NASBA, first described by Kievits *et al*. (1991), involves the simultaneous use of three enzymes, avian myeloblastosis virus reverse transcriptase, RNasemH, and T7 RNA polymerase. NASBA has been applied for the detection of *Campylobacter* (Vandervliet *et al.,* 1993). Uyttendaele *et al*. (1995) demonstrated the applicability of NASBA to the detection of *C. jejuni* in chicken skin and ground beef. *C. jejuni* could be detected to less than 10 cfu/10 g. However, these methods are very expensive and therefore not a feasible option for use in developing countries.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

Reference strains including *Campylobacter jejuni* ATCC 33291, *C. jejuni* ATCC 81176, *C. coli* MUMT 18407, *C. coli* MUMT 18630, *C. lari* ATCC 43675, *C. upsaliensis* DMST 19055, and *C. fetus* ATCC 27374 were used. All *Campylobacter* were cultured on modified charcoal cefoperazone desoxycholate agar (mCCDA) (Oxoid, Basingstoke, UK). The plates were incubated at 42°C for 24 h at microaerobic atmosphere with 5% O_2, 10% CO_2, and 85% N_2. *Campylobacter* were maintained in brain heart infusion broth (BHIB) (Difco, Detroit, USA) supplemented with 15% glycerol at -70°C. Other bacterial strains were used for the validation of the developed method including were cultured overnight in BHIB at 37°C. A wide range of bacteria was tested to verify possible cross-reactions (**Table 2**).

3.2 A novel *Campylobacter* apparatus for the detection of viable thermophilic *Campylobacter*.

3.2.1 Design of *Campylobacter* apparatus

The apparatus was designed based on the ability of *Campylobacter* to move through a membrane filter in a viscous condition. The apparatus was composed of two compartments connecting by a filter-containing division. One compartment was for the inoculation of food samples while the other compartment was filled with *Campylobacter* enrichment broth containing oxygen-reducing agent. This apparatus was a closed system for the isolation of *Campylobacter* without gas-generating sachet. Diagram of *Campylobacter* apparatus was shown in **Figure 1**.

Table 2. *Campylobacter* reference strains and other bacterial strains used for the validation of the established multiplex PCR.

Campylobacter reference strains (*n* = 7)	Gram-positive (*n* = 11)	Gram-negative (*n* = 26)	
Campylobacter jejuni ATCC 33291	*Arcanobacterium pyogenes*	*Achromobacter denitrificans*	*Providencia alcalifaciens*
Campylobacter jejuni ATCC 81176	*Bacillus cereus*	*Achromobacter xylosoxidans*	*Providencia stuartii*
Campylobacter coli MUMT 18407	*Bacillus subtilis*	*Acinetobacter baumanii*	*Pseudomonas aeruginosa*
Campylobacter coli MUMT 18630	*Corynebacterium diphtheriae*	*Acinetobacter haemolyticus*	*Salmonella Paratyphi*
Campylobacter lari ATCC 43675	*Corynebacterium xerosis*	*Acinetobacter lwoffii*	*Salmonella Typhimurium*
Campylobacter upsaliensis DMST 19055	*Enterococcus faecalis*	*Aeromonas hydrophila*	*Shigella dysenteriae*
Campylobacter fetus ATCC 27374	*Enterococcus faecium*	*Arcobacter butzleri*	*Shigella flexneri*
	Listeria monocytogenes	*Burkholderia cepacia*	*Shigella sonnei*
	Staphylococcus aureus	*Edwardsiella tarda*	*Vibrio alginolyticus*
	Staphylococcus saprophyticus	*Enterobacter cloacae*	*Vibrio vulnificus*
	Streptococcus group D	*Escherichia coli*	
		Helicobacter pylori	
		Klebsiella pneumoniae	
		Moraxella cartarrhalis	
		Morganella morganii	
		Proteus mirabilis	

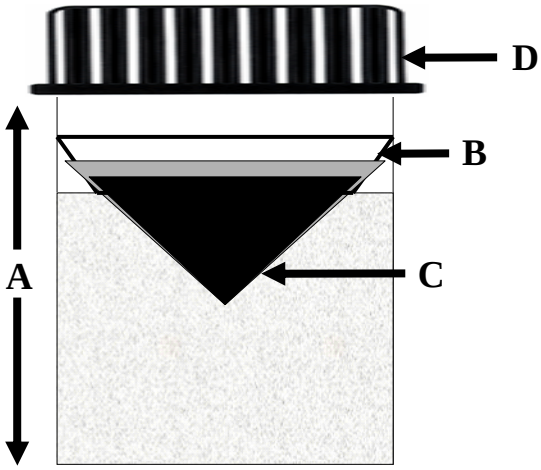

Figure 1. Diagram of *Campylobacter* apparatus. Enrichment chamber (NL1201-0100; outside diameter, 62 mm; internal diameter, 53 mm; length, 70 mm) (Nalgene Co., Rochester, N.Y.) containing 90 ml of Bolton broth (A). A polypropylene funnel (NL4250-0055; maximum diameter, 60 mm; minimum diameter, 35 mm; length, 20 mm) (Nalgene) placed over the chamber (B). A cone-shaped nitrocellulose membrane filter (0.45 µm) coating with Bolton broth containing 0.5% agar, filled with 2 ml of Bolton broth without antibiotics (C). A lid was loosely fitted on the container (D).

3.2.2 Optimization of *Campylobacter* apparatus

Nitrocellulose membrane filters with 0.45 μm and 0.65 μm pore size (Millipore, Watford, UK) were selected for this experiment. The membrane filter folded into a cone-shape was autoclaved. The sterile filter paper was submerged for 1 min in Bolton broth (Oxoid) containing 0%, 0.5%, and 1% agar (Difco) at 50°C. The filter paper was removed from the medium and placed in an upright position on a sterile petridish for 30 min to produce a soft-agar-coated membrane filter.

This apparatus with viable and non viable *Campylobacter* strains in the presence of background microbiota were evaluated. Each of the reference strains of *Campylobacter* was inoculated into mCCDA and incubated at 42°C for 24 h. After incubation, a loopful of bacteria was taken and suspended in 1 ml of buffer peptone water (BPW). Ten ml of viable inoculum were added to 90 ml of Bolton broth supplemented with 10 mg/l of amphotericin B, 20 mg/l of trimethoprim, 20 mg/l of vancomycin, and 32 mg/l of cefoperazone (Sigma-Aldrich, St. Louis, USA) in a sterile polypropylene plastic container to give the final cell numbers at 10-10^4 cfu/ml.

For a non viable inoculum control, viable bacterial cells at 10^4 cfu/ml were resuspended in 1 ml of saline containing 200 ppm sodium hypochlorite and incubated at room temperature for 4 h to kill all viable cells (Hayashi *et al*., 2006). Subsequently, the cell suspension was washed three times with saline and finally resuspended in 10 ml of BPW. Ten ml of non viable inoculum were added to 90 ml of Bolton broth supplemented with antibiotics in a sterile polypropylene plastic container to give the final cell numbers at 10^4 cfu/ml. Other bacterial species including *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis*, *Salmonella Paratyphi*, and *S.* Typhimurium were used as mixed microbiota. Each of the bacterial species was grown in Mueller-Hilton broth (Difco) at 37°C for 5 h and adjusted to a concentration approximately 10^4 cfu/ml. The bacterial inoculum was pooled together and prepared as described previously to give the final cell numbers at 10^4 cfu/ml.

A sterile polypropylene funnel with soft-agar-coated membrane filter was placed over the container. Two ml of Bolton broth was added into the cone, then a lid was loosely fitted over the container. The containers were then incubated at 37°C and 42 °C for 24 h. To determine the present of viable *Campylobacter*, 1 ml of the culture in the cone was determined by a drop plate method.

From each culture, 10 µl of the samples were transferred to modified charcoal desoxycholate agar (mCDA). Plates were allowed to dry and incubated at 42°C for 24 h. A typical colony of *Campylobacter* on mCDA has a gray, moistening, and effuse appearance.

3.2.3 Evaluation of *Campylobacter* apparatus for isolation of *Campylobacter* from artificially-inoculated chicken samples

Inoculum preparation was carried out as described previously. Twenty five g of chicken with or without 10 ml of the viable bacterial suspension were added to 90 ml of Bolton broth supplemented with antibiotics in a sterile plastic container. The initial numbers of artificially-inoculated viable cells per g was approximately $10\text{-}10^4$. The prepared samples were then processed and incubated in the same manner as described in previous experiment. Except, the containers were incubated at 37°C for 24 h or 37°C for 4 h, followed by further incubation at 42°C for 20 h as described by the US Food and Drug Authority (US FDA, 2001).

To determine the number of viable *Campylobacter*, 1 ml of the culture in the cone was determined by a drop plate method. One hundred µl of the bacterial suspension in each serial dilution was pipetted into a tube containing 900 µl of sterile BPW. From each dilution, 10 µl of the samples were transferred to mCCDA supplemented with antibiotics. Plates were allowed to dry and incubated at 42°C for 18-24 h.

3.2.4 Comparison of efficiency between *Campylobacter* apparatus and conventional method for the detection of *Campylobacter* from chicken samples

A total of 305 chicken samples including fresh chicken meat, chilled ready-to-eat products, and frozen chicken meat were tested. The samples were obtained from retail shops and supermarkets in Hat Yai, Songkhla, Thailand. All chicken samples are usually commercially sold as parts carcasses including breasts, legs, and wings.

A conventional method for the isolation of *Campylobacter* from food was performed according to Bacteriological Analytical Manual established by US FDA (US FDA, 2001). For analysis, 25 g slices were cut aseptically from the surface of the sample, at least 10 cm^2 of the skin was included. The sample was placed in a stomacher bag with 100 ml of Bolton broth supplemented with antibiotics and pummeled in a stomacher

HG400V (Mayo, Verona, Italy) for 1 min at 200 rpm. The isolation of *Campylobacter* was carried out according to the procedure in the previous report (Hunt *et al*., 1998). In brief, the sample in the enrichment broth was incubated at 37°C for 4 h, followed by further incubation at 42°C for 24 h. After 24 h, 10 µl loopfuls of sample homogenates were streaked on mCCDA supplemented with 10 mg/l of amphotericin B and 32 mg/l of cefoperazone. The identification of *Campylobacter* was carried out by the observation on colony characteristics, microscopic morphology, and biochemical tests including oxidase reaction, catalase production, and nalidixic acid sensitivity (Nachamkin, 2003).

3.3 A multiplex PCR for identification of thermophilic *Campylobacter*

3.3.1 Genomic DNA extraction

Total bacterial DNA was extracted by a modified phenol method (Sambrook and Russell, 2001). Five ml of bacterial suspension in Bolton broth was centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in 0.5 ml saline and vortex. Equal volume of cold phenol was added, invertedly mixed, and spun at 5,000 rpm at ambient temperature for 5-10 min. The aqueous phase containing nucleic acid was drawn and added into 0.6 ml of cold isopropanol. Proteins that separated into the organic phase or lay at the phase interface were discarded. DNA was precipitated, washed with 70% ethanol, and resuspended in 50 µl of Tris-Acetate-EDTA buffer (pH 8.0). The genomic DNA was quantified with a spectrophotometer. Extracted DNA was stored at 4°C for use.

3.3.2 Multiplex PCR

Two different multiplex PCR reactions were used in this study (**Table 3**). Set-A multiplex PCR was designed to detect four genes of 23S rRNA, *ceuE* encoding for siderophore transport, *glyA* for serine hydroxymethyltransferase, and *cdtB* for cytolethal-distending toxin. PCR was performed in a total reaction volume of 25 µl containing 1×PCR buffer (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, Hanover, USA), 1.0 U *Taq* polymerase (Fermentas), 50 ng 23S rDNA primers for *Campylobacter* (Eyers *et al.*, 1993), 150 ng and 100 ng *ceuE* primers for *C. jejuni* and *C. coli*, respectively (Houng *et al.*, 2001), 150 ng *cdtB* primers for *Campylobacter* (Bang *et al.*, 2003), 50 ng each of *glyA* primers for *C. lari* and

C. upsaliensis (Wang *et al.,* 2002). Either 4.8 µl of cultured *Campylobacter* prepared by simple boiling method or 2 µl of genomic DNA extracted with phenol were used as PCR templates. Template DNA was initially denatured at 94°C for 20 min. PCR amplification consisted of 30 cycles of 94°C for 1 min, 50-60°C for 1 min, and 72°C for 1 min. Final extension was determined at 72°C for 10 min. PCR cycles were carried out in PTC-100, Peltier thermal cycler (Pegasus Scientific, Maryland, USA). Two µl amplified products were analysed by 2% agarose (Gibco-BRL Life Technologies, Gaithersburg, USA) gel electrophoresis in Tris-Acetate-EDTA buffer at 100 V for 35 min. PCR products were visualized after ethidium bromide staining.

Set-B multiplex PCR detected five genes of 16S rRNA, *ceuE*, *gyrA* encoding for type II topoisomerase, *lpxA* for UDP-*N*-acetylglucosamine acyltransferase, and *cdtB* in one tube. PCR was performed in a total reaction volume of 25 µl as in Set-A, 150 ng 16S rDNA primers for *Campylobacter* (Linton *et al.,* 1996), 100 ng *gyrA* primers for *C. jejuni* (this study), 100 ng *ceuE* primers for *C. coli* (Houng *et al.,* 2001), 100 ng *lpxA*-F primers, and 150 ng *lpxA*-R primers for *C. lari* and *C. upsaliensis* (Klena *et al.* 2004), 150 ng *cdtB* primers for *Campylobacter* (Bang *et al.,* 2003) were incorporated. PCR amplification was carried out as in Set-A, except for annealing at 40-50°C for 1 min. Amplicons were detected by gel electrophoresis in the same manner as described in Set-A.

3.3.3 Specificity and sensitivity of the multiplex PCR

To test the specificity of the primers, PCR amplifications were performed on DNA isolated from the reference strains. A total of 2 µl of DNA extract was amplified in a 25 µl reaction volume. *Campylobacter* reference strains were used as positive controls and sterile water as a negative reagent control.

The sensitivity of the multiplex-PCR method was tested using both DNA and whole cell suspension. The sensitivity was investigated by extracting DNA from diluted pure cultures. DNA templates were prepared for the analysis of 100, 75, and 50 ng per PCR. For whole cell, *Campylobacter* were inoculated into mCCDA and incubated at 42°C for 24 h. After incubation, a loopful of the culture was suspended in 1 ml normal saline. Cell density was determined by spectrophotometer at 625 nm (OD 0.08-0.1) to give approximately 2×10^8 cfu/ml. A 10-fold serially diluted culture from 2×10^8-2×10^5 cfu/ml was tested. Each of the dilutions was 10-fold diluted in 0.075% Triton X-100 (Sigma-

Table 3. List of specific primers, conditions and sizes of amplicons used in this study.

	Target microbe /Specific for	Target gene	Annealing temperature (^{o}C)	Amplicon size (base pair)	References
Set-A	*Campylobacter*	23S rRNA	54	222	Eyers *et al.,* 1993
	Campylobacter	*cdtB*	42	495	Bang *et al.,* 2003
	C. jejuni	*ceuE*	55	783	Houng *et al.,* 2001
	C. coli	*ceuE*	55	645	Houng *et al.,* 2001
	C. lari	*glyA*	59	251	Wang *et al.,* 2002
	C. upsaliensis	*glyA*	59	204	Wang *et al.,* 2002
Set-B	*Campylobacter*	16S rRNA	55	816	Linton *et al.,* 1996
	Campylobacter	*cdtB*	42	495	Bang *et al.,* 2003
	C. jejuni	*gyrA*	51	290	This study
	C. coli	*ceuE*	55	645	Houng *et al.,* 2001
	C. lari	*lpxA*	50	233	Klena *et al.,* 2004
	C. upsaliensis	*lpxA*	50	206	Klena *et al.,* 2004

Aldrich) and centrifuged at 5,000 g for 5 min. 4.8 µl of the cell suspension was used directly in the PCR, resulting in 2×10^6 -2×10^3 cells per PCR.

3.3.4 Comparison of efficiency between multiplex PCR and biochemical method for the identification of *Campylobacter*

Seventy eight *Campylobacter* isolates from clinical and chicken caeca samples were conventionally cultured using membrane filter method (Steele and McDermott, 1984). Thirty three strains were isolated from faeces of patients with acute diarrhoea. Forty five strains were isolated from chicken caeca samples. Each sample was homogenized in Bolton broth. A pasteur pipette was used to place 8-10 drops of the sample onto the surface of a nitrocellulose membrane 0.65 µm pore size which was placed blood agar plate. The pores allowed the relatively slender *Campylobacter* to pass through. The membrane was left on the agar surface until all fluid passed through (20-30 min). The plates were incubated under microaerobic conditions for 2 days or up to 5 days for less common, slower growing species.

The identification of *C. jejuni* was performed by hippurate hydrolysis assay. Bacterial colonies were collected from mCCDA and shaken in 0.5 ml 1% sodium hippurate solution (Sigma-Aldrich). After 2 h in water bath at 37°C, 0.2 ml of ninhydrin solution (Sigma-Aldrich) was added on the top of the hippurate solution in each tube. Further incubation at 37°C was carried out for 10 min for colour development. A positive test was recorded as deep purple colour, crystal violet-like, indicating the presence of glycine with resulted from hippurate hydrolysis. A pale purple colour or colourless tubes were considered negative.

3.4 Enhancement of viable *Campylobacter* detection by chemotactic stimuli

3.4.1 Preparation of fresh mucin-bile constituents and chemicals

Fresh gall-bladder from a cow was placed on ice and immediately transported to the laboratory. Bile was removed from the gall-bladder with a sterile syringe. The organ was opened and rinsed with cold distilled water, and the inside surface mucosa was scraped off with a glass slide to removed mucin which was added to the bile. A portion (1:1) was sterilized by filtration through a 0.22 µm syringe filter (Acrodisk, Texas, USA)

and stored at -4°C. Amino acids including L-aspartate, L-cysteine, histidine, phenylalanine, and tryptophan (Sigma-Aldrich) were used in this study. Sodium salts including sodium acetate, sodium citrate, and sodium pyruvate were obtained from Mallinckrodt (Phillipsburg, New Jersey, USA). All chemicals were prepared in phosphate-buffered saline (pH 7.4) at the concentration of 0.001-0.1 M.

3.4.2 Assays of motility ability in viscous condition

Each *Campylobacter* reference strain was inoculated onto mCCDA and incubated at 42°C for 24 h. After incubation, a few colonies were taken and suspended in 1 ml BPW. Ten ml of the viable inoculum were added to 90 ml Bolton broth in a sterile polypropylene plastic container to give the final cell numbers range from 10-10^4 cfu/ml.

The apparatus was designed based on the ability of *Campylobacter* to move through a 0.45 µm pore size in a viscous condition as described previously. In brief, nitrocellulose membrane filter with 0.45 µm pore size folded into a cone-shape was autoclaved. The tested chemical and mucin-bile constituents were added into Bolton broth containing 0.5% agar. The sterile filter paper was submerged for 1 min in a soft-agar-coated membrane filter at 50°C. The filter paper was removed from the medium and placed in an upright position on a sterile petridish for 30 min to produce a soft-agar-coated membrane filter.

A sterile polypropylene funnel with soft-agar-coated membrane filter was placed over the container. Two ml of Bolton broth was added into the cone, then a lid was loosely fitted over the container. The containers were then incubated at 37°C and 42°C for 6, 12, 18, and 24 h. To determine the numbers of viable *Campylobacter*, 1 ml of the culture in the cone was determined by drop plate method. One hundred µl of the bacterial suspension in each serial dilution was pipetted into a tube containing 900 µl sterile BPW. From each dilution, 10 µl of the samples were transferred to mCCDA. Plates were allowed to dry and incubated at 42°C for 18-24 h. All experiments were carried out twice and measurements were performed in triplicate.

3.4.3. Evaluation of chemotactic effects on the numbers of viable *Campylobacter* cells isolated from artificially-inoculated chicken samples

Inoculum preparation was carried out as described previously. Twenty five g of chicken with 10 ml of the viable or injured bacterial suspension were added to 90 ml Bolton broth supplemented with antibiotics in a sterile plastic container. The initial numbers of artificially-inoculated viable cells per g was approximately $10\text{-}10^4$. The prepared samples were then processed and incubated in the same manner as described in previous experiment.

3.4.4 Estimation of viable but non-culturable (VBNC) cell numbers

The involvement of bacterial starved cells during exposure to the simulated aquatic environments at 4°C was investigated. Inoculum preparation was carried out as described previously. The changes in viable cell numbers were investigated by drop plate method. The confirmation of viable but non-culturable state of *Campylobacter* was studied by observation on microscopic morphology as described by Bovill and Mackey (1997). To a 1 ml sample, 0.1 ml formaldehyde solution (37%) was added and the sample was left for 20 min. A drop was then applied to a microscope slide, air-dried and fixed with methanol for 2 min. It was then Gram-stained using carbol fuchsin as the counter-stain. Approximately 1,000 cells were then counted, noting the type as vibrioid or coccoid, using a microscope equipped with a x100 oil immersion objective lens.

3.5 Application of *Campylobacter* apparatus for the isolation of *Campylobacter concisus* and *Helicobacter pylori* from gastric biopsies

3.5.1 Evaluation of *Campylobacter* apparatus for isolation of *C. concisus* and *H. pylori*

The apparatus was evaluated with viable *C. concisus* or *H. pylori* strains in the presence of background microbiota. Bacterial strains including *Bacteroides fragilis, Campylobacter concisus* GC 99, *Clostridium perfringens, Enterococcus faecalis, Escherichia coli, Helicobacter pylori* GC 11, *H. pylori* GC 17, and *H. pylori* GC 51 were grown on Columbia blood agar base (Fluka, Buchs, Switzerland) with 15% defibrinated

horse blood (Sigma-Aldrich, Seelze, Germany) under microaerophilic conditions at 37°C for 24 h. After incubation, a loopful of *C. concisus* or *H. pylori* strains was suspended in 1 ml of sterile BPW. Ten ml of viable inoculum were added to 90 ml of Bolton broth in a sterile polypropylene plastic container to give the final cell numbers at 10-10^4 cfu/ml. Other bacteria were used as mixed microbiota. Each of bacterial inoculum at 10^4 cfu/ml was pooled together and prepared as described previously to give the final cell numbers at 10^4 cfu/ml.

Campylobacter apparatus was prepared as described previously. The containers were then incubated at 37°C for 1, 2, 3, and 4 day. To determine the numbers of viable cells, a drop plate method were used as described previously.

3.5.2 Conventional method for the isolation of *C. concisus* and *H. pylori* from gastric biopsy specimens

A total of 5 gastric biopsies were collected from Centre for Digestive Disease at Sydney, Australia. Biopsy specimens were homogenised and incubated in 1.5 ml micro test tubes with BHIB containing 2.0 µg/ml of amphotericin B, 2.5 mg/ml of trimethoprim, 5.0 mg/ml of vancomycin, and 1,250 U/ml of polymixin B (Sigma-Aldrich) at 37°C for 72 h. After 72 h, the sample was streaked on Columbia blood agar base with 15% defibrinated horse blood and 5 mg/ml vancomycin. The plates were incubated at 37°C for 72 h at microaerobic atmosphere as described previously. The identification of *C. concisus* and *H. pylori* was carried out by the observation on colony characteristics, motility and morphology under phase-contrast microscopy, and biochemical tests including oxidase reaction, catalase, and urease production.

3.6 Statistical analysis

Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences package version 8 for windows (SPSS, Chicago, USA).

CHAPTER 4

RESULTS

4.1 A novel apparatus for the detection of viable thermophilic *Campylobacter*

In this study, the development of *Campylobacter* apparatus on the basis of the motility ability of the organism for the detection of viable thermotolerant *Campylobacter* was established. Regarding pore sizes of filter, nitrocellulose membrane filters with 0.45 μm and 0.65 μm pore sizes were evaluated. Moreover, agar-coated filter with different concentrations, 0.0, 0.5, and 1.0% were compared. When the inoculum was 10^4-10^2 cfu/ml, viable *C. jejuni* ATCC 33291 was recovered by both pore sizes filters coating with 0.5 and 1.0% agars (**Table 4**). When the inoculum was 10 cfu/ml, only 0.5% agar-coated filters recovered *C. jejuni*, whereas 1.0% agar-coated filter could not detect low levels of viable cells. Coating with 0.5% agar, background microbiota were not detected by 0.45 μm pore size filter. However, *C. jejuni* was overgrown by microbiota with 0.65 μm pore size filter at 37°C. In addition, uncoated filters showed no viable cells detection even at 10^4 cfu/ml.

Cultures with nonviable cells were not recovered inside the cone of *Campylobacter* apparatus, indicating that dead cells (without motility ability) did not pass through the agar-coated membrane filter at all concentrations. Similar patterns were observed with other species including *C. coli*, *C. lari*, and *C. upsaliensis* (**Table 5-9**). Therefore, 0.45 μm pore sizes filter coating with 0.5% agar was selected for further study.

The effect of temperature on the growth of *C. jejuni* ATCC 33291 in the apparatus was investigated (**Figure 2**). *C. jejuni* moved through the soft-agar-coated filter at both 37°C and 42°C. However, the numbers of the organisms at 42°C were higher than those at 37°C at any point of time. Following the inoculation of *C. jejuni* (10^4 cfu/ml) in the enrichment chamber, the highest concentration of the cells reached 10^5 cfu/ml at 37°C and 10^7 cfu/ml at 42°C, after 24 h inoculation. When the inoculation was 10 cfu/ml, the numbers of viable cells at 37°C was 10^2 cfu/ml and 10^4 cfu/ml, at 42°C after 24 h inoculation. The mean generation time at 37°C was 2.15 h while it was 1.35 h at 42°C.

Table 4. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter jejuni* ATCC 33291 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

Table 5. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter jejuni* ATCC 81176 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

Table 6. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter coli* MUMT 18407 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

Table 7. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter coli* MUMT 18630 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

Table 8. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter lari* ATCC 43675 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

Table 9. Optimization of *Campylobacter* apparatus for the detection of *Campylobacter upsaliensis* DMST 19055 in the presence of background microbiota.

Inoculum (cfu/ml)	0.0% Agar				0.5% Agar				1.0% Agar			
	0.45 µm		0.65 µm		0.45 µm		0.65 µm		0.45 µm		0.65 µm	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
Viable cells												
10^4	-	-	-	-	+	+	+	+	+	+	+	+
10^3	-	-	-	-	+	+	+	+	+	+	+	+
10^2	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-
Nonviable cells												
10^4	-	-	-	-	-	-	-	-	-	-	-	-
Mixed microbiota												
10^4	-	-	-	-	-	-	+[a]	-	-	-	-	-

After the inoculation of *Campylobacter* into the enrichment chamber, the apparatus was incubated for 24 h. Viable counts were determined on modified charcoal desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions. +, growth of *Campylobacter*. +[a], growth of microbiota. -, no growth.

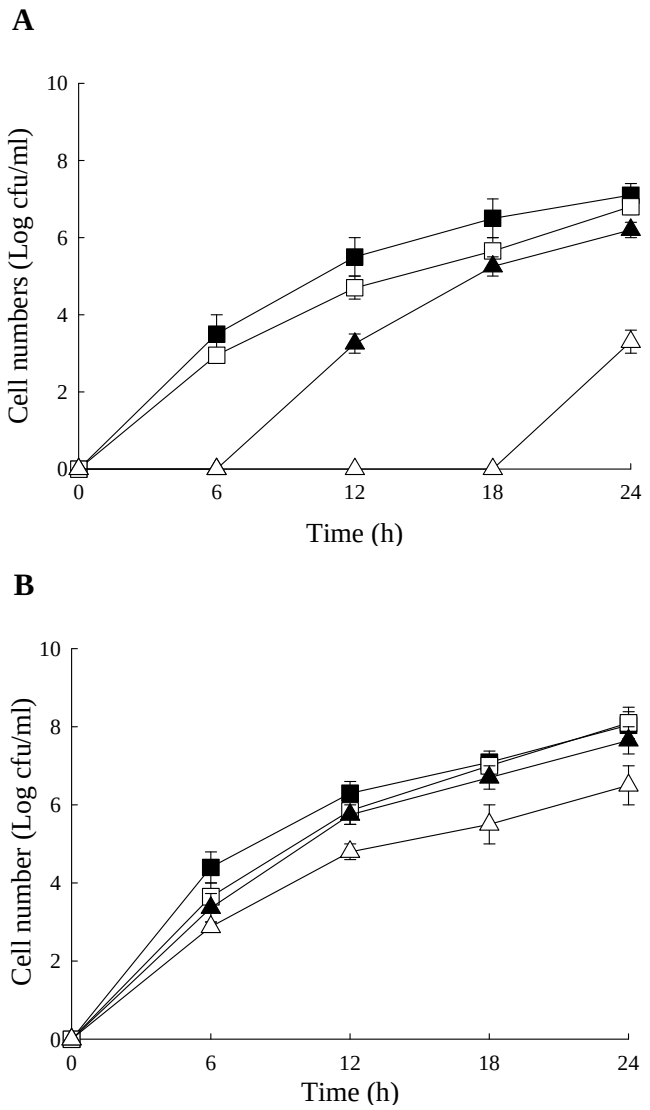

Figure 2. The effect of temperature on the recovery of *Campylobacter jejuni* ATCC 33291 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

Similar effects were observed with other species including *C. coli, C. lari,* and *C. upsaliensis* (**Figure 3-7**).

The model of this apparatus obtained from the pure culture studies was applied for the isolation of *Campylobacter* from artificially-inoculated food samples. All chicken samples artificially inoculated with viable *Campylobacter* cells yielded positive results (**Figure 8-10**). The sensitivity of this apparatus was tested with serially diluted pure cultures. The results shown in **Figure 8-10** indicated that this method was able to detect the presence of 10 bacterial cells per g for all *Campylobacter*. The reliable incubation time required for the detection of the inoculated viable strain was at least 6 h irrespective of initial inoculum.

Standard conventional cultural method was used for the comparative evaluation of the *Campylobacter* apparatus (**Table 10**). The results demonstrated that 14 of 305 samples (4.59%) collected were positive for *Campylobacter*. From these samples, seven isolates from fresh chicken samples and seven isolates from frozen chicken samples were recovered. All isolates were biochemically identified as *C. coli*. With the apparatus, *Campylobacter* were detected from 31 of 305 samples (10.16%). Sixteen isolates from fresh chicken samples and 15 isolates from frozen chicken samples were recovered. Twenty-eight (9.18%) and 3 (0.98%) isolates were identified as *C. coli* and *C. jejuni*, respectively. The results from **Table 10** clearly demonstrated that the *Campylobacter* apparatus yielded overall higher recovery rate than a conventional method.

All samples that were positive by conventional assay were also positive by apparatus. Additional nine samples were detected as positive by the apparatus but not found by conventional culture. *C. coli* was the most common species detected in this study. It was isolated from 93.33% (42/45) of the positive samples. In contrast, *C. jejuni* was isolated from only 6.67% (3/45).

4.2 A multiplex PCR for the identification of thermophilic *Campylobacter*

The objective of the present investigation was to establish a multiplex PCR for the identification of these four species and compare with conventional cultural methods. We also evaluated different DNA template preparation methods to achieve high detection rate of *Campylobacter*. In addition, *cdtB* gene-based multiplex PCR in this study can simultaneously detect the presence of *cdtB* gene.

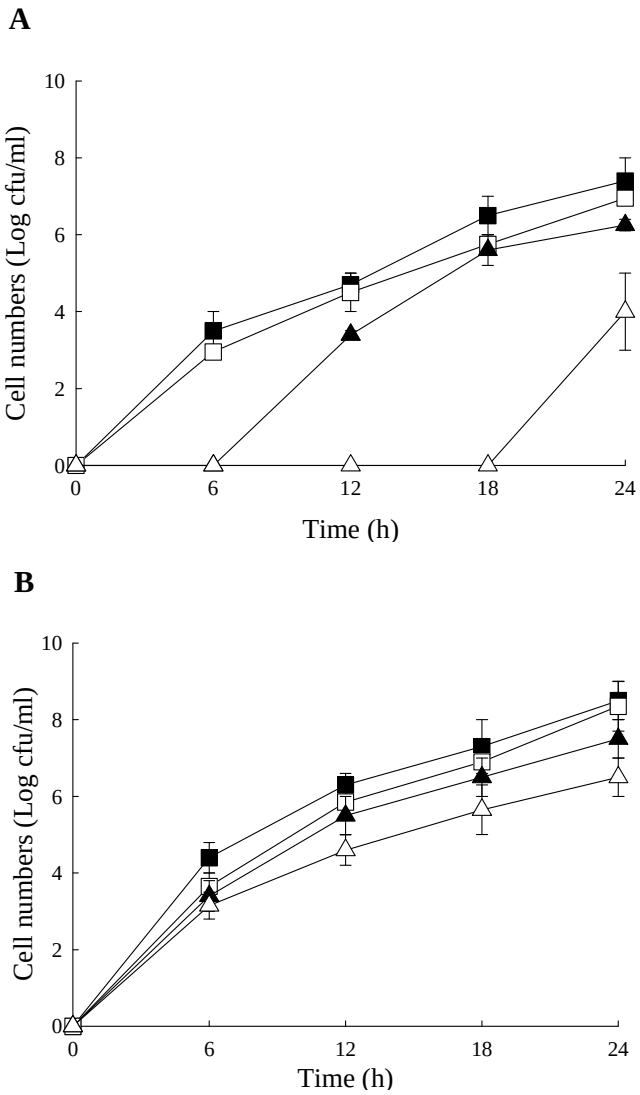

Figure 3. The effect of temperature on the recovery of *Campylobacter jejuni* ATCC 81176 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

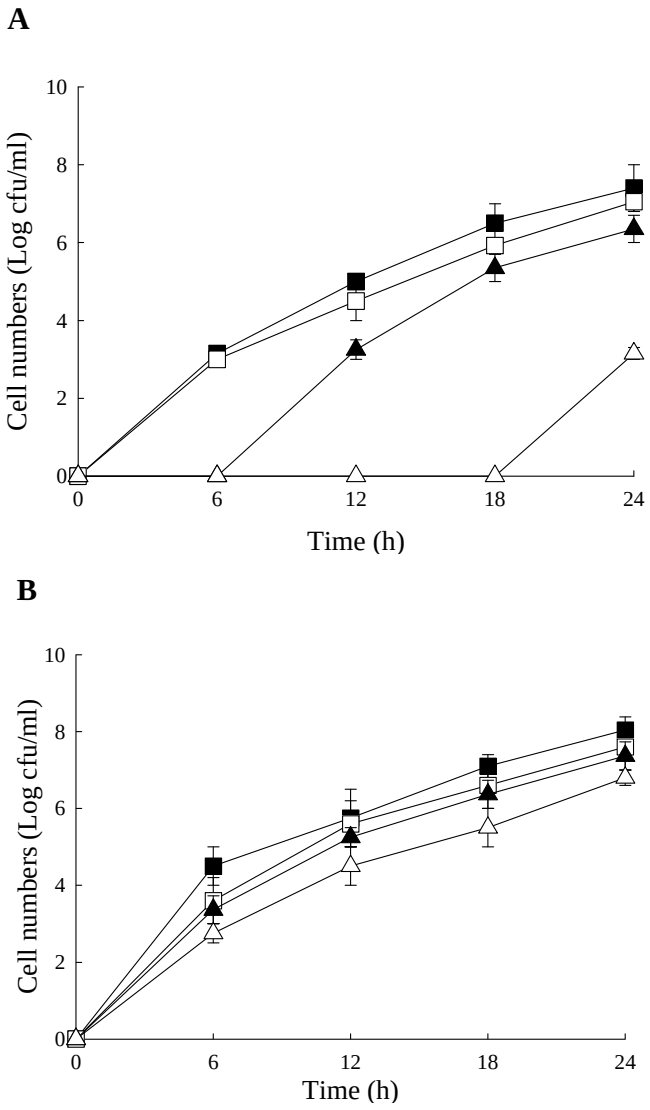

Figure 4. The effect of temperature on the recovery of *Campylobacter coli* MUMT 18407 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

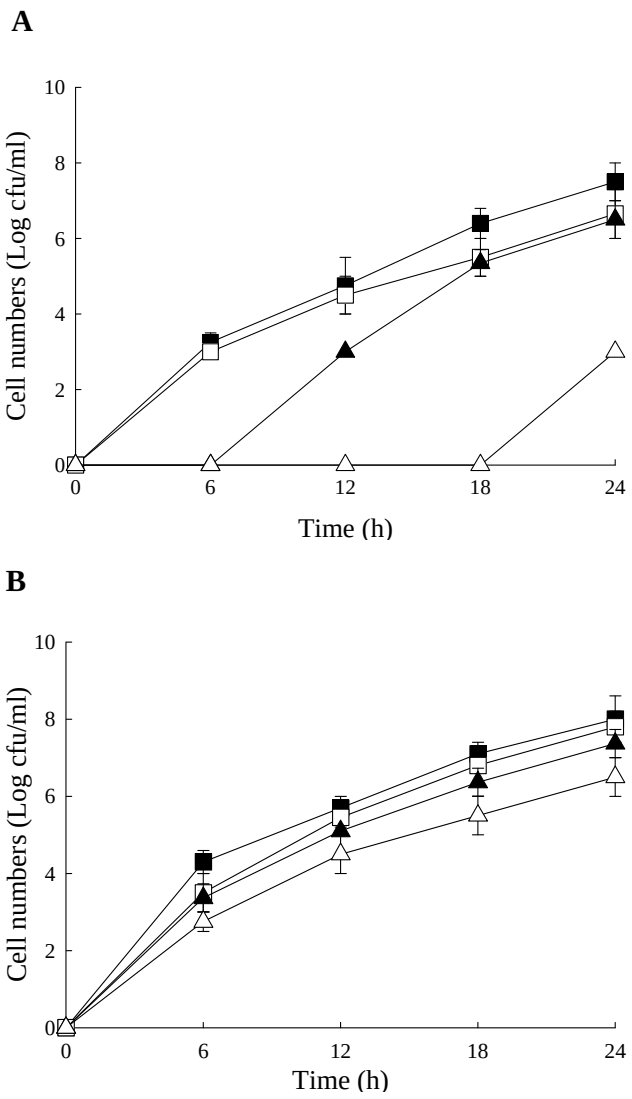

Figure 5. The effect of temperature on the recovery of *Campylobacter coli* MUMT 18630 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

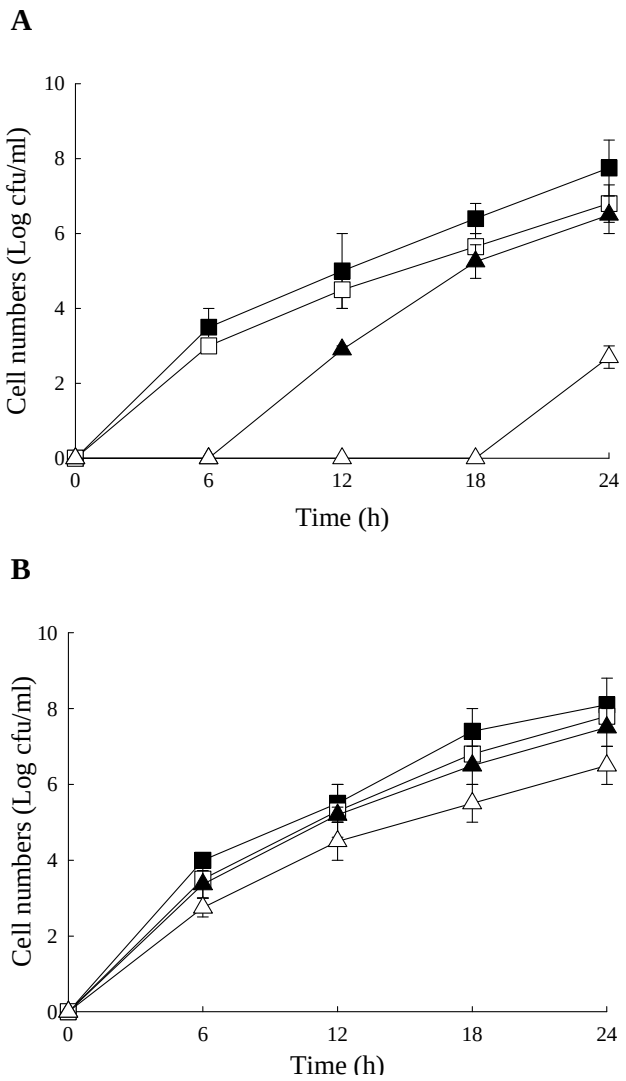

Figure 6. The effect of temperature on the recovery of *Campylobacter lari* ATCC 43675 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

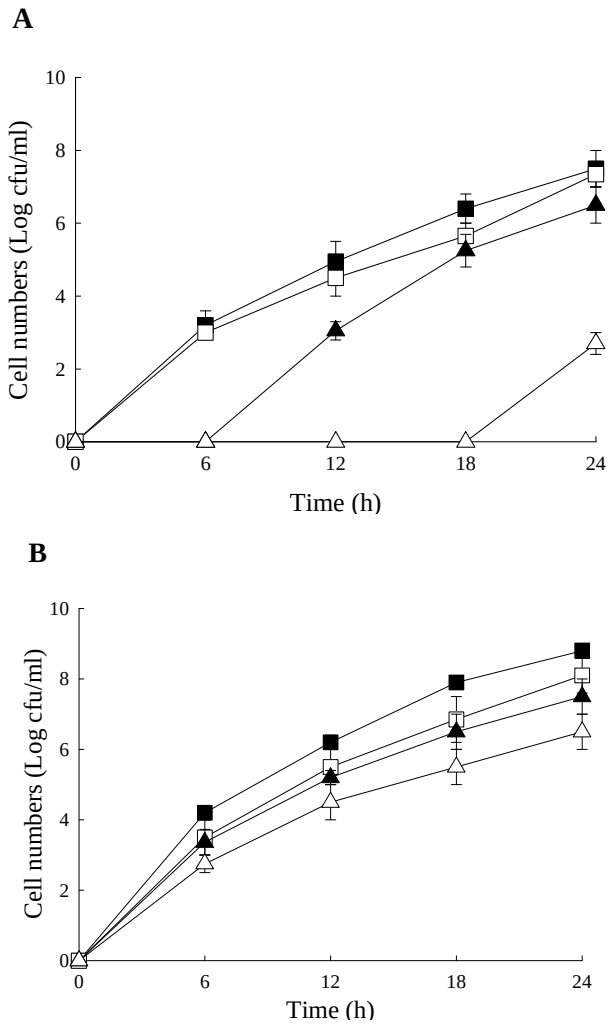

Figure 7. The effect of temperature on the recovery of *Campylobacter upsaliensis* DMST 19055 in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation of *C. jejuni* into the enrichment chamber, the apparatus was incubated at 37°C (A), and 42°C (B). Viable counts were determined on modified charcoal cefoperazone desoxycholate agar. The plates were incubated at 42°C for 24 h under microaerobic conditions.

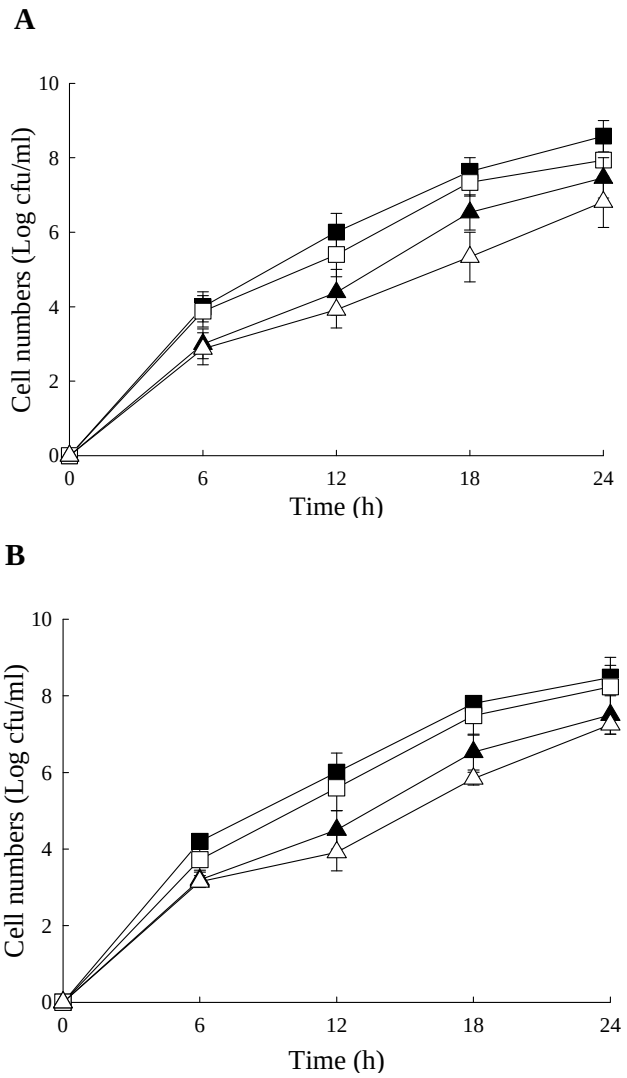

Figure 8. The recovery of *Campylobacter jejuni* ATCC 33291 (A) and *Campylobacter jejuni* ATCC 81176 (B) at 42°C isolated from artificially-inoculated chicken. Various concentrations of viable bacteria (10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g) were added to fresh chicken meat. Viable counts were made on modified charcoal cefoperazone desoxycholate agar, incubated at 42°C for 24 h under microaerobic conditions.

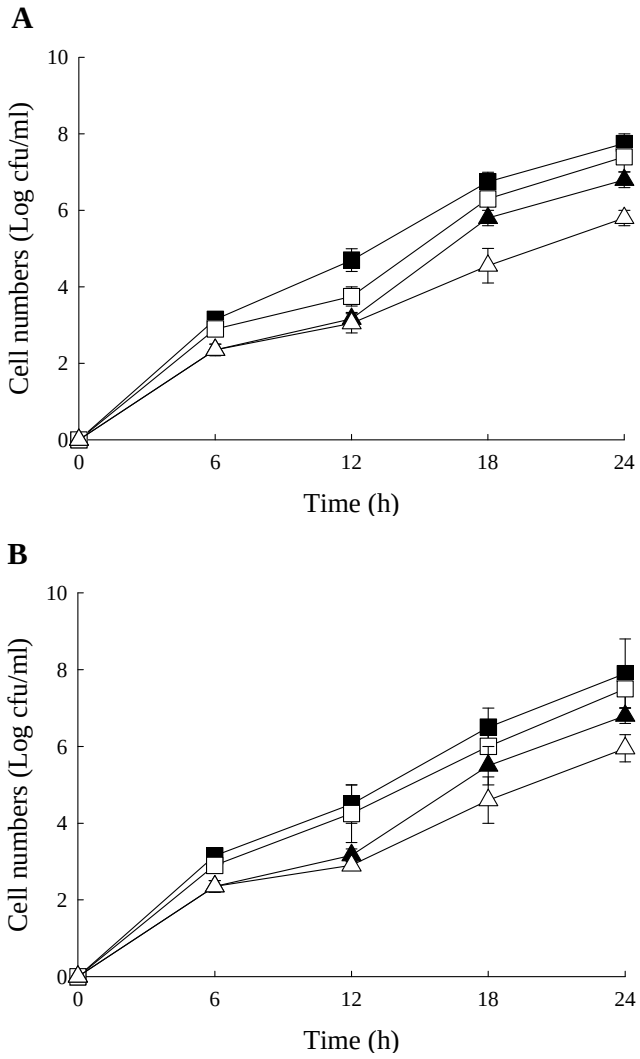

Figure 9. The recovery of *Campylobacter coli* MUMT 18407 (A) and *Campylobacter coli* MUMT 18630 (B) at 42°C isolated from artificially-inoculated chicken. Various concentrations of viable bacteria (10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g) were added to fresh chicken meat. Viable counts were made on modified charcoal cefoperazone desoxycholate agar, incubated at 42°C for 24 h under microaerobic conditions.

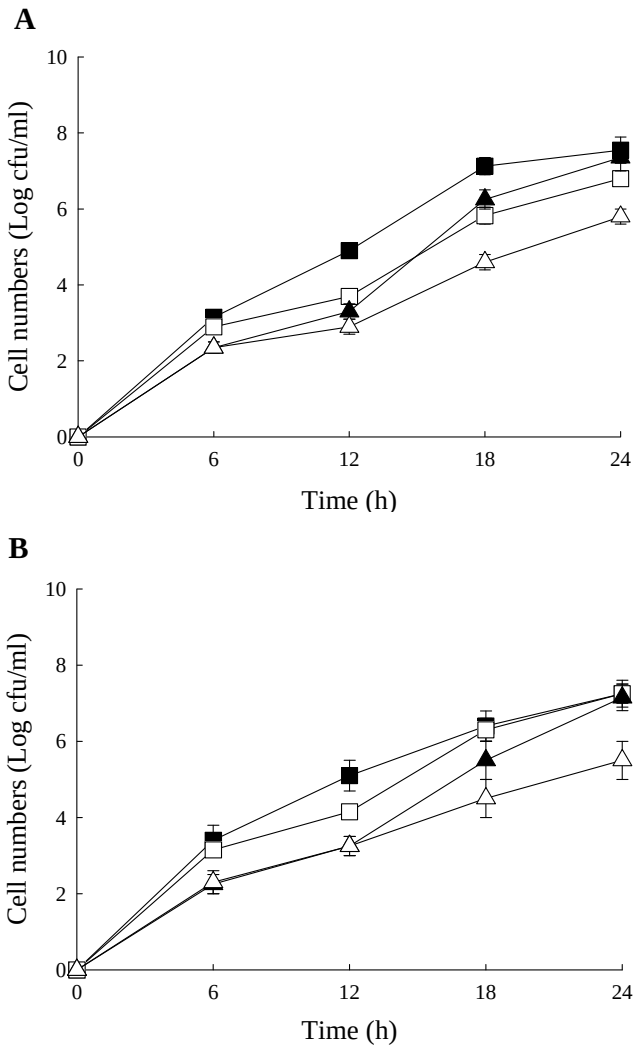

Figure 10. The recovery of *Campylobacter lari* ATCC 43675 (A) and *Campylobacter upsaliensis* DMST 19055 (B) at 42°C isolated from artificially-inoculated chicken. Various concentrations of viable bacteria (10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g) were added to fresh chicken meat. Viable counts were made on modified charcoal cefoperazone desoxycholate agar, incubated at 42°C for 24 h under microaerobic conditions.

Table 10. Comparison of *Campylobacter* positive samples from chicken samples by *Campylobacter* apparatus and conventional method.

Chicken samples	Methods	Numbers of positive samples (%)	Numbers of positive samples from (%)			Numbers of samples identified as (%)		
			breast	leg	wing	*C. coli*	*C. jejuni*	
Fresh samples (n=150)	Apparatus	16 (10.67)	4 (2.67)	6 (4.00)		6 (4.00)	14 (9.33)	2 (1.33)
	Conventional	7 (4.67)	1 (0.67)	2 (1.33)	4 (2.67)	7 (4.67)	0	
Processed samples (n=155)								
Ready-to-eat (n=50)	Apparatus	0	0	0		0	0	0
	Conventional	0	0	0		0	0	0
Frozen meat (n=105)	Apparatus	15 (14.29)	4 (3.81)	6 (5.71)		5 (4.00)	14 (13.33)	1 (0.95)
	Conventional	7 (6.67)	2 (1.91)	3 (2.86)		2 (1.91)	7 (6.67)	0
Total (n=305)	Apparatus	31 (10.16)	8 (2.62)	12 (3.93)		11 (3.61)	28 (9.18)	3 (0.98)
	Conventional	14 (4.59)	3 (0.98)	5 (1.64)		6 (1.97)	14 (4.59)	0

Two sets of primers were selected for the identification of *C. jejuni, C. coli, C. lari,* and *C. upsaliensis*. Multiplex PCR was carried out in a single tube method by incorporating all the four-species specific primers. The assay was standardized by optimizing the annealing temperature that resulted in the generation of differently sized species specific amplicons. The optimal condition of set-A multiplex PCR was performed using a reaction mixture including six primer pairs. The combination of 23S rDNA, *ceuE*, and *glyA* primers were able to detect four-species specific bands at 50-60°C annealing temperatures. When adding *cdtB* primers, *C. jejuni*-specific bands of *ceuE* turned to be negative.

For set-B, the combination of 16S rDNA, *gyrA*, *ceuE*, and *lpxA* primers were able to detect four-species specific bands at 40-50°C annealing temperatures. When adding *cdtB* primers, *C. jejuni*-specific, *C. coli*-specific, *C. upsaliensis*-specific, and *C. fetus*-specific bands were also detected at the annealing temperatures. The specific bands were clearest with no ghost bands appeared at 50°C annealing temperature. Therefore, this temperature was selected for set-B multiplex PCR. Varied concentrations of *Taq* polymerase between 1.0-1.5 U was examined in the reaction. High concentration of *Taq* polymerase did not enhance the amplification of some targets. The results indicated that set-B multiplex PCR with 50°C annealing temperature and 1.0 U *Taq* polymerase were able to effectively amplify specific products.

The species-specific primers were tested against purified chromosomal DNA isolated from the reference strains of thermotolerant *Campylobacter*, nonthermotolerant *Campylobacter*, and non-*Campylobacter* isolates. **Figure 11** shows the results of multiplex PCR from *Campylobacter* cultures. This multiplex PCR generated products with a length of 816 bp amplicon for 16S rRNA gene from all the *Campylobacter*, 645 bp amplicon from *C. coli* for the *ceuE* gene, and 290 bp amplicon from *C. jejuni* for the *gyrA* gene. For the *lpxA* gene, 233 bp and 206 bp amplicons were identified as *C. lari* and *C. upsaliensis*, respectively. Specificity was tested with different thermophilic *Campylobacter*. All of the reference strains reacted with their respective specific primers set to generate a PCR amplicon, while *C. fetus*, a nonthermotolerant produced only 16S rDNA amplicon. When all four species of *Campylobacter* were mixed in one sample, four-species specific bands were clearly observed in the PCR profile. The multiplex PCR was tested on pure culture of various non-*Campylobacter* strains. Nonspecific product was observed with *Moraxella catarrhalis*, a very light band was observed around 233 bp.

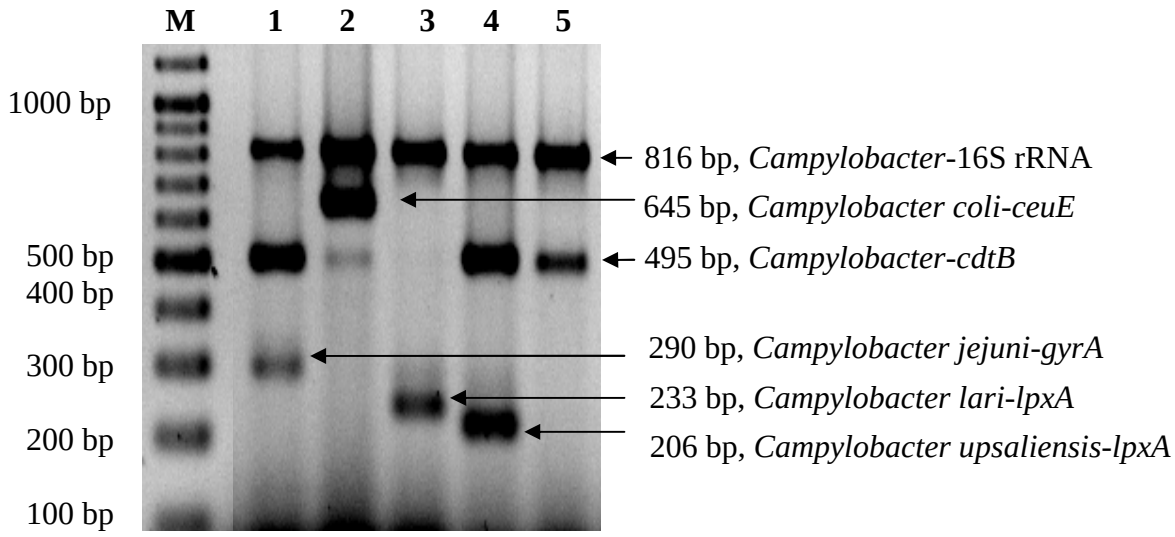

Figure 11. Multiplex PCR analysis of *Campylobacter* reference strains. Lane 1, *Campylobacter jejuni* ATCC 33291; lane 2, *Campylobacter coli* MUMT 18407; lane3, *Campylobacter lari* ATCC 43675; lane 4, *Campylobacter upsaliensis* DMST 19055; lane 5, *Campylobacter fetus* ATCC 27374. Lane M: 100 bp DNA ladder (size marker).

However, this result did not affect the validity of the multiplex PCR because of *M. catarrhalis* were unable to grow at 42°C.

The sensitivity of the multiplex PCR was tested with both DNA and whole-cell suspensions. The sensitivity was investigated by extracting DNA from serially diluted pure cultures. The multiplex PCR was performed on serially diluted genomic DNA from all *Campylobacter* reference strains (**Figure 12**). The lowest amount of genomic DNA required in order to yield sufficient PCR product to be detected by this multiplex PCR was 100 ng. Whole-cell suspensions were prepared for the analysis of 10^6, 10^5, 10^4, 10^3, 10^2, and 10 cells per PCR (**Figure 13**).

In these experiments, cell suspensions were held in a boiling water-bath to lyse the bacteria and the cell lysates were used as template DNA in the PCR reaction. Only *C. coli* reference strains failed to show the expected PCR product, suggesting that these isolates might be resistant to lysis by boiling in water. To improve detection, a lysate was initially denatured at 94°C for 20 min in 0.075% Triton X-100, and then directly used as template for PCR amplification reaction. The result clearly indicated that this direct multiplex PCR was able to detect the presence of 2×10^5 cfu per PCR. It is to be noted that a weak signal was observed at 2×10^3 cells for *C. upsaliensis*.

A total of 78 isolates including 33 clinical isolates from human origin and 45 isolates from chicken caeca were comparatively identified by conventional method and multiplex PCR detection (**Table 11**). Hippurate hydrolysis, 59 (75.6%) and 19 (24.4%) were identified as *C. jejuni* and *C. coli*, respectively. Multiplex PCR was carried out to further confirm the species of *Campylobacter* strains. Of the 78 specimens, 55 (70.5%) were identified as *C. jejuni*, 18 (23.0%) as *C. coli*, and 5 (6.5%) as a mixed infection of both species which was impossible to differentiate conventionally since their colonies were very similar.

PCR fragments of the *cdtB* gene with the expected sizes of 495 bp, were observed in 62 (84.9%) isolates. *cdtB* was detected in 100% and 38.9% of C. *jejuni* and *C. coli*, respectively (**Table 12**). In this study, the *cdtB* gene was present in 100% of *C. jejuni* isolates tested. A similar frequency was observed on both human and chicken isolates. In contrast, this gene was found in 33% and 44% of *C. coli* isolated from human and chickens, respectively.

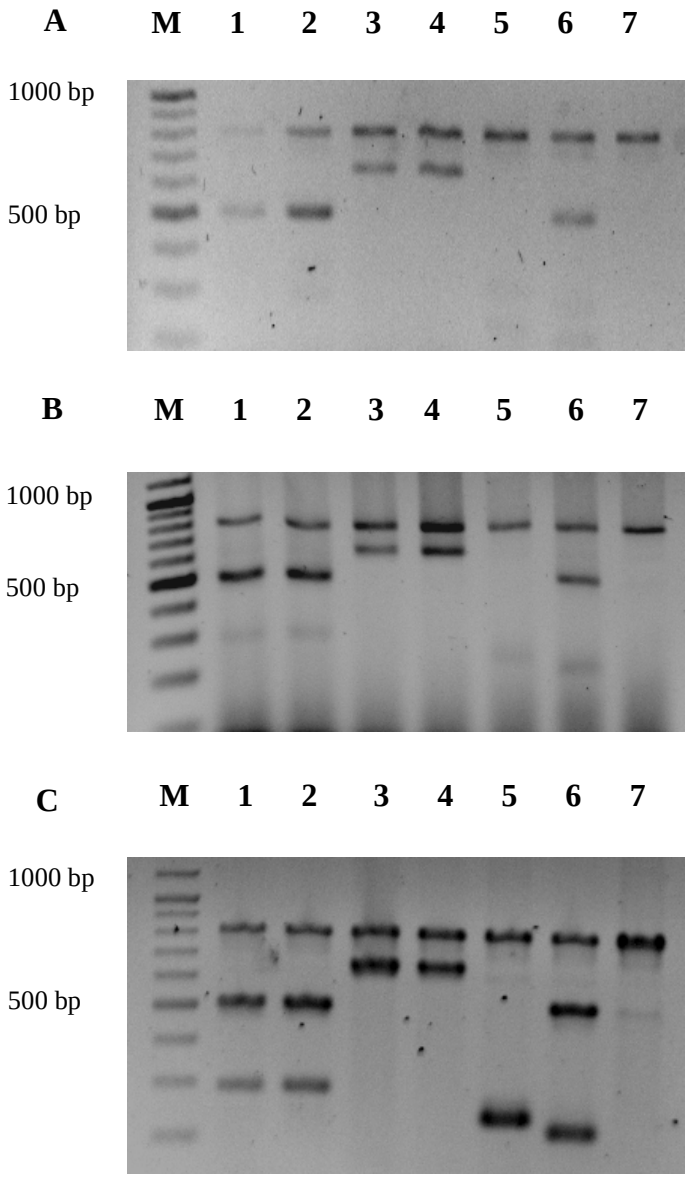

Figure 12. Multiplex PCR sensitivity study by dilutions of bacterial and derived from pure culture. Lane 1, *Campylobacter jejuni* ATCC 33291; lane 2, *Campylobacter jejuni* ATCC 81176; lane 3, *Campylobacter coli* MUMT 18407; lane 4, *Campylobacter coli* MUMT 18630; lane 5, *Campylobacter lari* ATCC 43675; lane 6, *Campylobacter upsaliensis* DMST 19055; lane 7, *Campylobacter fetus* ATCC 27374. Lane M: 100 bp DNA ladder (size marker)**.** DNA concentrations: 50 ng (A), 75 ng (B), and 100 ng (C).

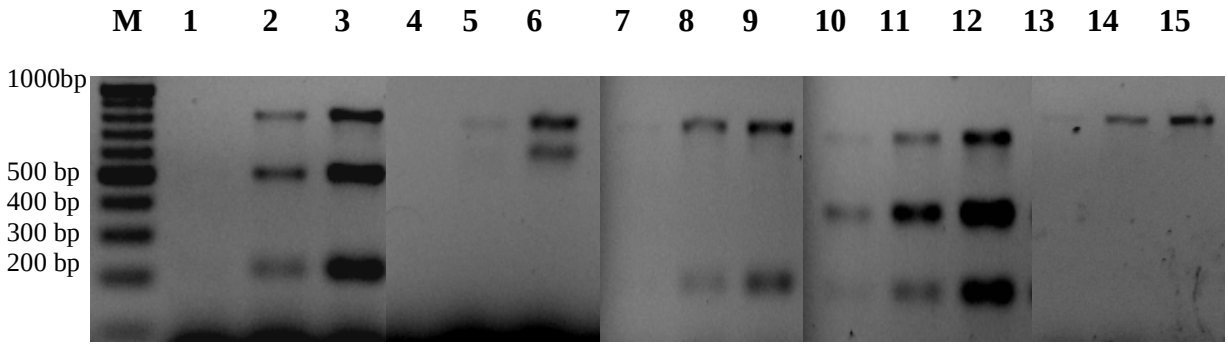

Figure 13. Multiplex PCR sensitivity study by 10-flod dilutions of bacterial cells. Lanes 1-3, *Campylobacter jejuni* ATCC 33291; lanes 4-6, *Campylobacter coli* MUMT 18407; lanes 7-9, *Campylobacter lari* ATCC 43675; lanes 10-12, *Campylobacter upsaliensis* DMST 19055; lanes 13-15, *Campylobacter fetus* ATCC 27374. Lane M: 100 bp DNA ladder (size marker). Bacterial cell concentrations: 2×10^3 cfu/ml (lane 1, 4, 7, 10, 13), 2×10^4 cfu/ml (lanes 2, 5, 8, 11, 14), and 2×10^5 cfu/ml (lanes 3, 6, 9, 12, 15).

Table 11. Comparison of species identification of *Campylobacter* isolated from human (n = 33) and chicken caeca (n = 45) samples by hippurate hydrolysis and multiplex PCR.

Sources of isolates		Numbers of species (%) identified by	
		Hippurate hydrolysis	Multiplex PCR
Human samples	*C. jejuni*	20 (25.6)	23 (29.5)
	C. coli	13 (16.7)	9 (11.5)
	Mixed	0	1 (1.3)
Chicken samples	*C. jejuni*	39 (50.0)	32 (41.0)
	C. coli	6 (7.7)	9 (11.5)
	Mixed	0	4 (5.2)
Total	*C. jejuni*	59 (75.6)	55 (70.5)
	C. coli	19 (24.4)	18 (23.0)

Table 12. Multiplex PCR detection of *cdtB* genes in *Campylobacter* isolated from human (n = 32) and chicken caeca (n = 41) samples.

	Frequency of *cdtB* genes detected (%)		
Species	Human samples	Chicken samples	Total
C. jejuni	23/23 (100)	32/32 (100)	55/55 (100)
C. coli	3/9 (33)	4/9 (44)	7/18 (38.9)

4.3 Enhancement of viable *Campylobacter* detection by chemotactic stimuli

In this study, the effects of various chemotactic stimuli on the motility ability of *Campylobacter* including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* to pass through a 0.45 µm pore size filter in viscous condition were investigated. Accumulation of viable bacteria inside the cone of *Campylobacter* apparatus resulted from movement in response to a gradient of attractant. Fresh mucin-bile constituents (1:1), varieties of amino acids, and sodium salts were tested in viscous condition for their effects on the chemotactic behaviour of the four strains of thermophilic *Campylobacter*. At both 37°C and 42°C, no significant differences of chemotactic effects on *C. jejuni* were observed by amino acids or sodium salts at the concentration of 0.001-0.1 M (**Table 13**).

Positive chemotaxis was observed towards mucin-bile constituents at the concentration of 1, 5, and 10% (**Table 13**). At 6 h, 10% mucin-bile constituents demonstrated a significant increase in the numbers of viable *C. jejuni* cells ($p < 0.05$). At 37°C, following the inoculation of 10^4 cfu/g, the cell numbers in the tested group were 10^6 cfu/ml while those in the control group were 10^4 cfu/ml. The highest concentration of cell numbers reached 10^8 cfu/ml in the tested group and 10^6 cfu/ml in control group at 24 h. Similar results were obtained with other concentrations of inocula. When the inoculation was 10 cfu/g, the numbers of cells reached 10^3 cfu/ml in the tested group, but no viable cells were detected in the control group after 6 h. The cell numbers in the tested group were 10^5 cfu/ml and 10^3 cfu/ml after 24 h inoculation, respectively (**Figure 14A, B**).

Similar effects on recovery of *C. jejuni* were observed at 42°C (**Figure 14C, D**), and also with other species including *C. coli*, *C. lari*, and *C. upsaliensis* (**Figure 15-16**). However, the numbers of the organisms at 42°C appeared to be higher than those at 37°C. After 24 h following the inoculation of 10 cfu/ml in the tested group, the cell numbers reached 10^5 cfu/ml at 37°C while they were approximately 10^6 cfu/ml at 42°C (**Figure 14A, C**).

Mucin-bile constituents was the only attractant that could enhance motility ability of *Campylobacter*, therefore it was further investigated whether it could support the growth of bacteria. Growth response following the inoculation of *C. jejuni* (10^4 cfu/ml) with the highest concentration (10%) of the attractant commonly employed in a chemotatic assay that resulted in positive chemotactic response is shown in **Figure 17**.

Table 13. Comparison of chemotactic effects by different chemical stimuli on *Campylobacter jejuni* ATCC 33291 isolated from artificially-inoculated chicken samples.

Chemicals	Numbers of viable cells (Mean ± SE)	
	37°C	42°C
L-aspartate (M)		
0.001	$2.33 \pm 0.53 \times 10^6$	$2.42 \pm 0.57 \times 10^7$
0.01	$1.88 \pm 0.44 \times 10^6$	$2.33 \pm 0.37 \times 10^7$
0.1	$1.48 \pm 0.32 \times 10^6$	$2.03 \pm 0.47 \times 10^7$
L- cysteine (M)		
0.001	$2.66 \pm 0.32 \times 10^6$	$2.53 \pm 0.55 \times 10^7$
0.01	$2.12 \pm 0.45 \times 10^6$	$2.44 \pm 0.15 \times 10^7$
0.1	$2.03 \pm 0.62 \times 10^6$	$2.23 \pm 0.33 \times 10^7$
Histidine (M)		
0.001	$2.51 \pm 0.55 \times 10^6$	$2.22 \pm 0.52 \times 10^7$
0.01	$2.44 \pm 0.55 \times 10^6$	$3.29 \pm 0.34 \times 10^7$
0.1	$1.81 \pm 0.55 \times 10^6$	$2.27 \pm 0.58 \times 10^7$
Mucin-bile constituents (%)		
1	$1.87 \pm 0.35 \times 10^8$	$3.26 \pm 0.88 \times 10^9$
5	$2.35 \pm 0.59 \times 10^8$	$2.45 \pm 0.73 \times 10^9$
10	$2.42 \pm 0.35 \times 10^8$	$1.88 \pm 0.38 \times 10^9$
Phenylalanine (M)		
0.001	$2.37 \pm 0.20 \times 10^6$	$2.31 \pm 0.22 \times 10^7$
0.01	$2.78 \pm 0.09 \times 10^6$	$2.50 \pm 0.24 \times 10^7$
0.1	$3.16 \pm 0.49 \times 10^6$	$2.33 \pm 0.72 \times 10^7$

Table 13. (Continued).

Chemicals	Numbers of viable cells (Mean ± SE)	
	37°C	42°C
Tryptophan (M)		
0.001	$2.47 \pm 0.68 \times 10^6$	$2.24 \pm 0.57 \times 10^7$
0.01	$2.03 \pm 0.62 \times 10^6$	$2.83 \pm 0.53 \times 10^7$
0.1	$2.73 \pm 0.55 \times 10^6$	$1.89 \pm 0.73 \times 10^7$
Sodium acetate (M)		
0.001	$2.33 \pm 0.27 \times 10^6$	$2.24 \pm 0.22 \times 10^7$
0.01	$2.63 \pm 0.55 \times 10^6$	$2.33 \pm 0.32 \times 10^7$
0.1	$3.31 \pm 0.45 \times 10^6$	$2.11 \pm 0.82 \times 10^7$
Sodium citrate (M)		
0.001	$2.58 \pm 0.33 \times 10^6$	$1.83 \pm 0.62 \times 10^7$
0.01	$1.58 \pm 0.33 \times 10^6$	$2.13 \pm 0.62 \times 10^7$
0.1	$1.85 \pm 0.33 \times 10^6$	$2.41 \pm 0.62 \times 10^7$
Sodium pyruvate (M)		
0.001	$2.13 \pm 0.61 \times 10^6$	$2.63 \pm 0.22 \times 10^7$
0.01	$2.35 \pm 0.71 \times 10^6$	$2.03 \pm 0.32 \times 10^7$
0.1	$2.22 \pm 0.51 \times 10^6$	$1.87 \pm 0.53 \times 10^7$
Control	$2.19 \pm 0.53 \times 10^6$	$3.12 \pm 0.53 \times 10^7$

Viable counts with initial inoculum of 10^4 cfu/g on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

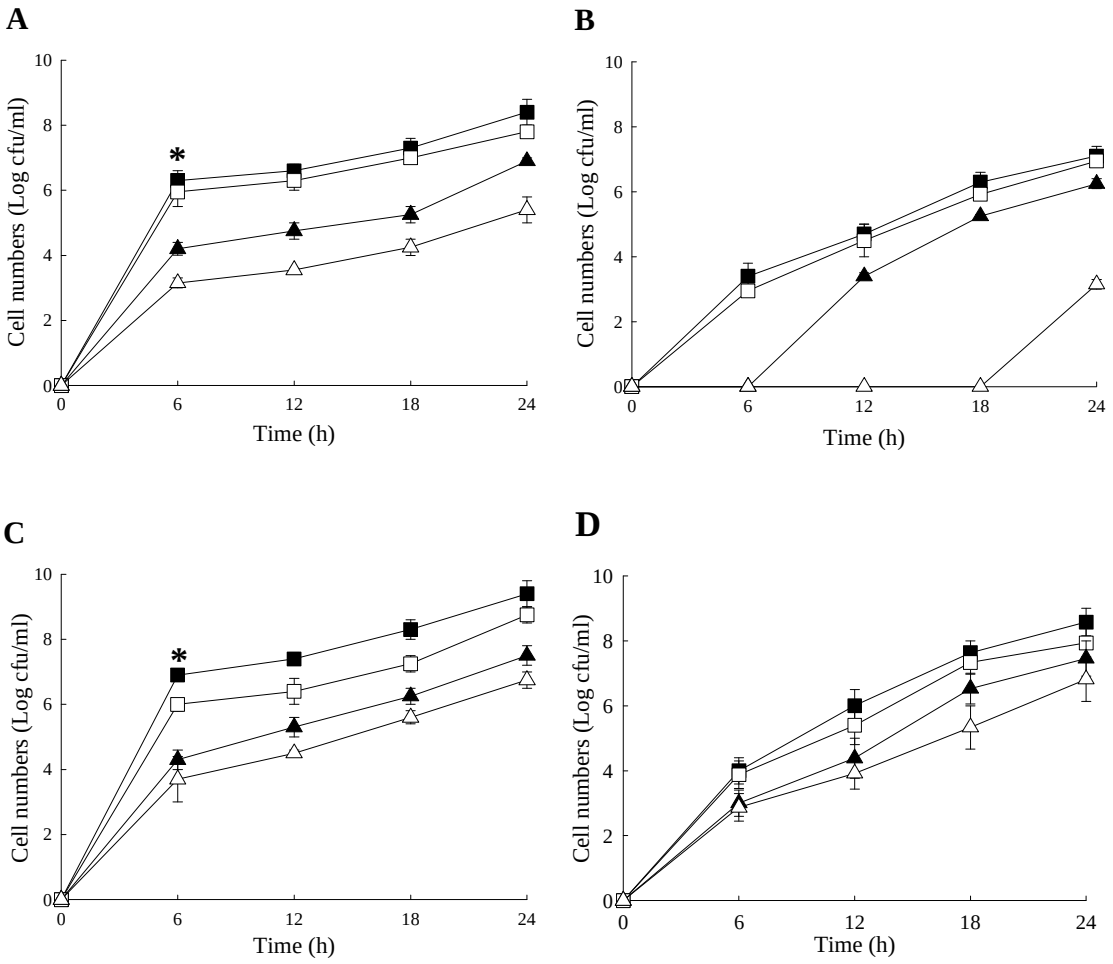

Figure 14. Chemotactic effects of 10% mucin-bile constituents on recovery of *Campylobacter jejuni* ATCC 33291 isolated from artificially-inoculated chicken samples. (A) Tested group at 37°C, (B) Control group without mucin-bile at 37°C, (C) Tested group at 42°C, and (D) Control group without mucin-bile at 42°C. Various concentrations of viable bacteria: 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g were added to fresh chicken meat. Viable counts were performed on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. An asterisk (*) indicates values significantly higher than those of the controls ($P < 0.05$).

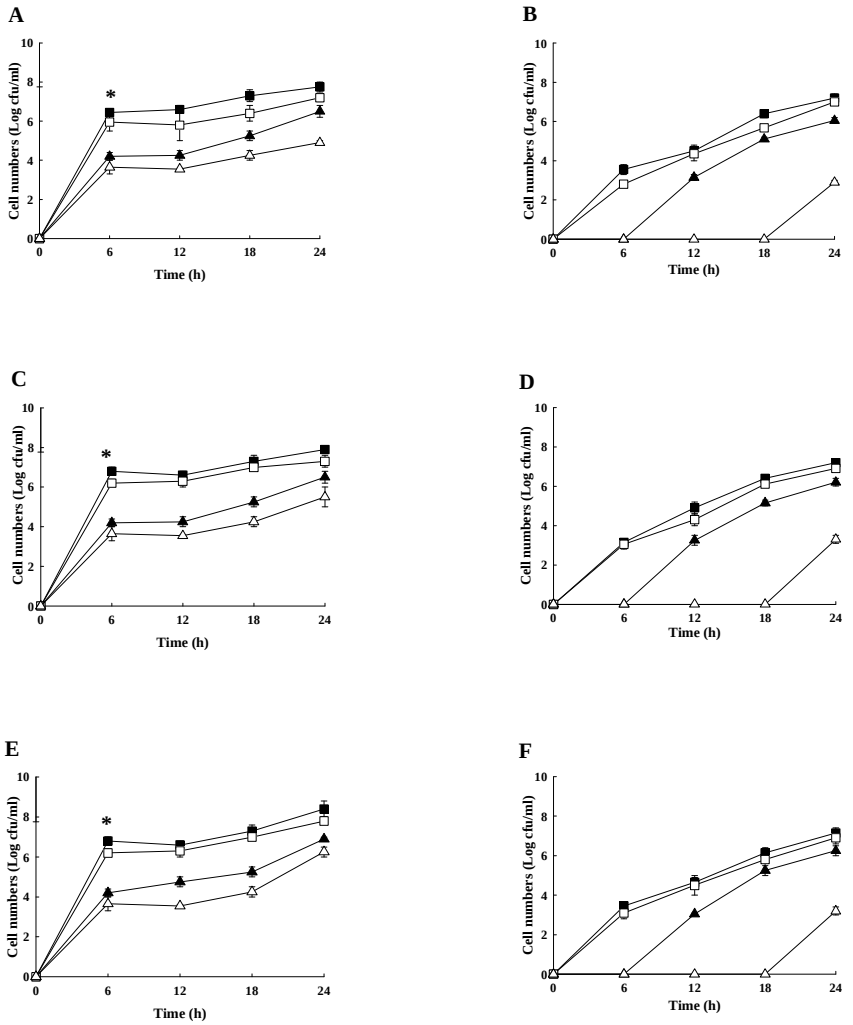

Figure 15. Chemotactic effects of 10% mucin-bile constituents on recovery of other thermophilic *Campylobacter* from artificially-inoculated chicken samples at 37°C. (A) *Campylobacter coli* MUMT 18407, (B) *Campylobacter coli* without mucin-bile, (C) *Campylobacter lari* ATCC 43675, (D) *Campylobacter lari* without mucin-bile, (E) *Campylobacter upsaliensis* DMST 19055, and (F) *Campylobacter upsaliensis* without mucin-bile. Various concentrations of viable bacteria: 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g were added to fresh chicken meat. An asterisk (*) indicates values significantly higher than those of the controls ($P < 0.05$).

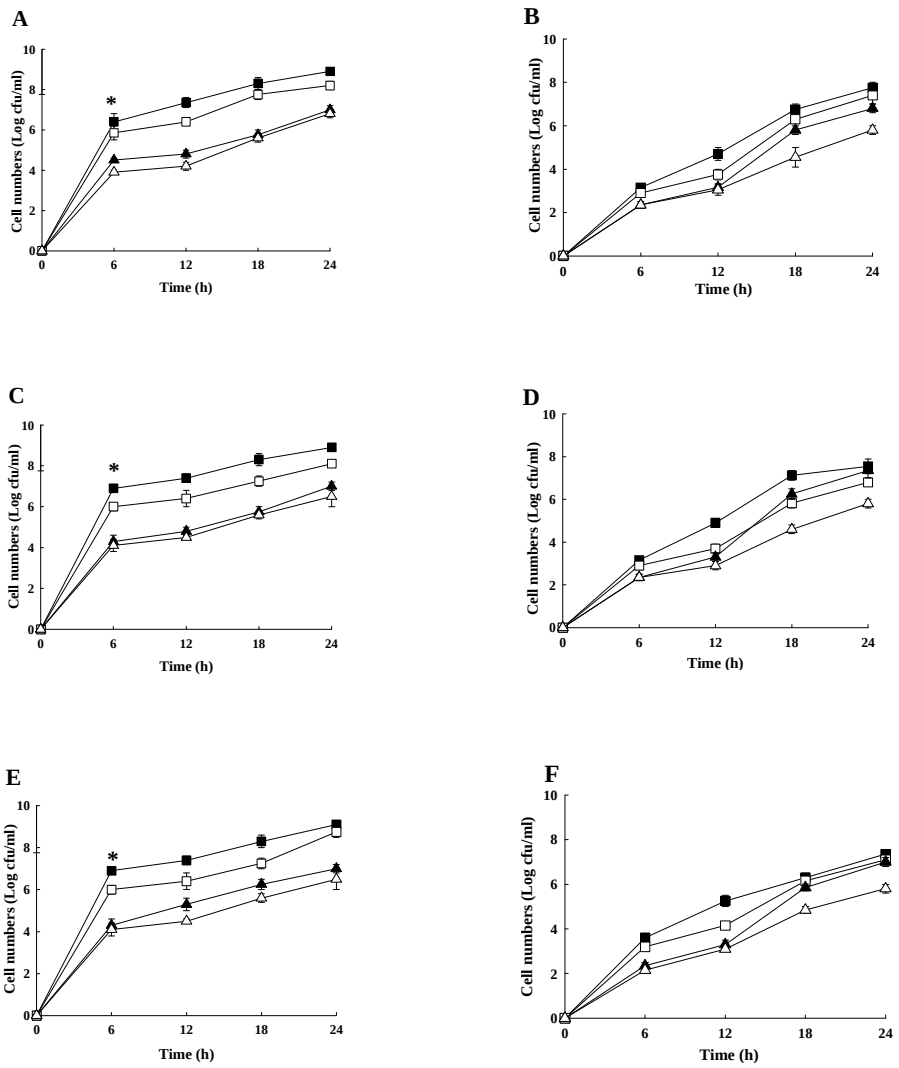

Figure 16. Chemotactic effects of 10% mucin-bile constituents on recovery of other thermophilic *Campylobacter* from artificially-inoculated chickens samples at 42°C. (A) *Campylobacter coli* MUMT 18407, (B) *Campylobacter coli* without mucin-bile, (C) *Campylobacter lari* ATCC 43675, (D) *Campylobacter lari* without mucin-bile**,** (E) *Campylobacter upsaliensis* DMST 19055, and (F) *Campylobacter upsaliensis* without mucin-bile. Various concentrations of viable bacteria: 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/g were added to fresh chicken meat. An asterisk (*) indicates values significantly higher than those of the controls ($P < 0.05$).

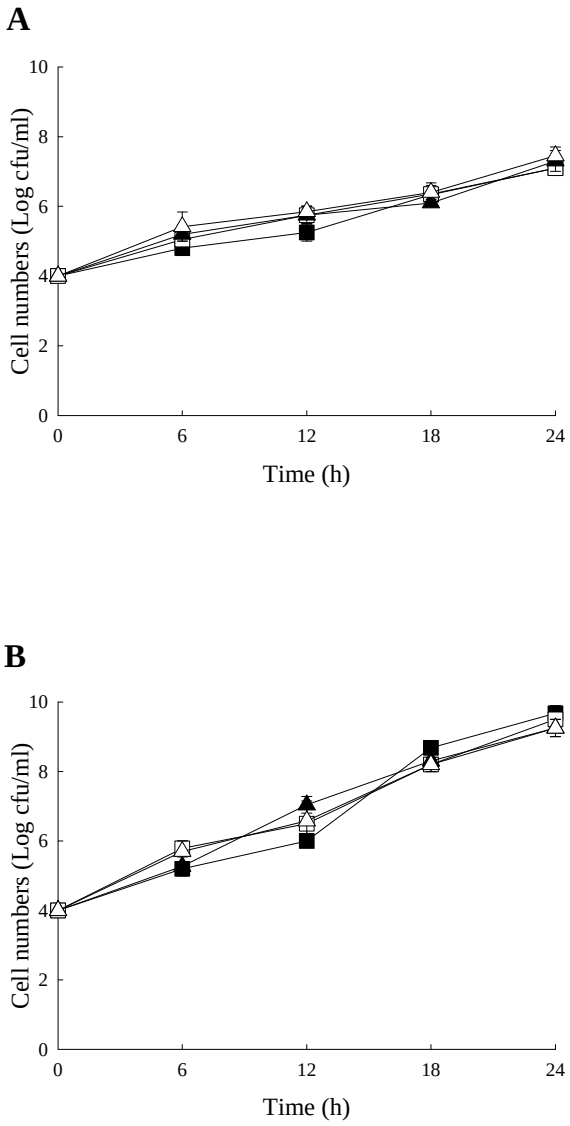

Figure 17. Effect of 10% mucin-bile constituents on growth of *Campylobacter jejuni* ATCC 33291 (■), *Campylobacter coli* MUMT 18407 (□), *Campylobacter lari* ATCC 43675 (▲), and *Campylobacter upsaliensis* DMST 19055 (Δ). After the inoculation of viable bacteria 10^4 cfu/ml in Bolton broth, the appendolf was incubated at (A) 37°C and (B) 42°C. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h.

The highest concentration of the cells reached 10^7 and 10^9 cfu/ml at 37°C and 42°C after 24 h inoculation, respectively. Lower concentrations of mucin-bile constituents at 1% and 5% demonstrated similar effects.

The numbers of viable cells were monitored following starvation. The numbers of viable cells decreased after 2 day starvation at 4°C. Cell counts for the four strains revealed that there were losses in culture viability of 1 log during the second day of incubation. A sudden decrease in culture viability of 2 logs was observed after 14 days of incubation (**Table 14**). Number of coccoid cells increased after 2 day starvation. In addition, percentages of coccoid cells increased to 89% and 92% after day 14 and 28, respectively. Similar results were observed in other thermophilic *Campylobacter* including *C. coli*, *C. lari*, and *C. upsaliensis* (**Table 15-17**). The starved cells showed lower chemotactic response to 10% mucin-bile constituents than non-starved cells significantly (**Table 18**).

4.5 Application of *Campylobacter* apparatus for the isolation of *Campylobacter concisus* and *Helicobacter pylori* from gastric biopsies

Following the inoculation of *C. concisus* GC 99 (10^4 cfu/ml) in the enrichment chamber, the highest concentration of the cells reached 10^5 cfu/ml, after 96 h inoculation. When the inoculation was 10^3 cfu/ml and 10^2cfu/ml, the numbers of viable cells were 10^4 cfu/ml and 10^3 cfu/ml, respectively (**Figure 18A**). However, *C. concisus* was not recovered at 10 cfu/ml. Similar patterns were observed with *H. pylori* GC 11, *H. pylori* GC 17, and *H. pylori* GC 51 (**Figure 18B-19**). As expected, *B. fragilis*, *C. perfringens*, *E. faecalis*, and *E. coli* were not detected inside the cone of the apparatus.

The sensitivity of this apparatus was tested with serially diluted pure cultures. The results indicated that this method was able to detect the presence of viable *C. concisus* and *H. pylori* with a minimal bacterial density of 10^2 cfu/ml. In addition, the reliable incubation time required for the detection of the inoculated viable strains was at least 72 h.

The model of this apparatus obtained from the pure culture studies was applied for the isolation of *C. concisus* and *H. pylori* from gastric biopsies. In total, 5 biopsies were comparatively studied using both conventional method and apparatus. Unfortunately, all samples were negative for *C. concisus* and *H. pylori*.

Table 14. Effects of duration of frozen storage at 4°C on the numbers of viable cells and coccoid cells of *Campylobacter jejuni* ATCC 33291.

Storage time (Days)	Numbers of viable cells (Mean ± SE)	Percentage of coccoid cells
0	$5.23 \pm 0.33 \times 10^4$	0
1	$3.47 \pm 0.65 \times 10^4$	0
2	$2.23 \pm 0.84 \times 10^3$	12
3	$2.28 \pm 0.25 \times 10^3$	17
4	$2.86 \pm 0.43 \times 10^3$	19
5	$2.73 \pm 0.38 \times 10^3$	22
6	$2.58 \pm 0.75 \times 10^3$	25
7	$2.26 \pm 0.15 \times 10^3$	29
14	$1.32 \pm 0.73 \times 10^2$	89
28	$1.23 \pm 0.22 \times 10^2$	92

After the inoculation of viable bacteria 10^4 cfu/g in chicken meat sample, the culture was kept at 4°C. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

Table 15. Effects of duration of frozen storage at 4°C on the numbers of viable cells and coccoid cells of *Campylobacter coli* MUMT 18407.

Storage time (Days)	Numbers of viable cells (Mean ± SE)	Percentage of coccoid cells
0	$4.28 \pm 0.22 \times 10^4$	0
1	$3.22 \pm 0.15 \times 10^4$	0
2	$3.33 \pm 0.66 \times 10^3$	10
3	$2.47 \pm 0.25 \times 10^3$	15
4	$2.75 \pm 0.43 \times 10^3$	20
5	$2.33 \pm 0.66 \times 10^3$	22
6	$2.22 \pm 0.75 \times 10^3$	24
7	$2.26 \pm 0.58 \times 10^3$	31
14	$1.63 \pm 0.33 \times 10^2$	91
28	$1.33 \pm 0.57 \times 10^2$	92

After the inoculation of viable bacteria 10^4 cfu/g in chicken meat sample, the culture was kept at 4°C. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

Table 16. Effects of duration of frozen storage at 4°C on the numbers of viable cells and coccoid cells of *Campylobacter lari* ATCC 43675.

Storage time (Days)	Numbers of viable cells (Mean ± SE)	Percentage of coccoid cells
0	$4.75 \pm 0.33 \times 10^4$	0
1	$3.47 \pm 0.40 \times 10^4$	0
2	$3.53 \pm 0.84 \times 10^3$	13
3	$3.86 \pm 0.55 \times 10^3$	17
4	$2.52 \pm 0.44 \times 10^3$	19
5	$2.47 \pm 0.38 \times 10^3$	23
6	$2.33 \pm 0.75 \times 10^3$	27
7	$2.44 \pm 0.25 \times 10^3$	29
14	$1.55 \pm 0.43 \times 10^2$	87
28	$1.62 \pm 0.35 \times 10^2$	93

After the inoculation of viable bacteria 10^4 cfu/g in chicken meat sample, the culture was kept at 4°C. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

Table 17. Effects of duration of frozen storage at 4°C on the numbers of viable cells and coccoid cells of *Campylobacter upsaliensis* DMST 19055.

Storage time (Days)	Numbers of viable cells (Mean ± SE)	Percentage of coccoid cells
0	$5.23 \pm 0.33 \times 10^4$	0
1	$4.44 \pm 0.75 \times 10^4$	0
2	$3.22 \pm 0.66 \times 10^3$	9
3	$2.28 \pm 0.25 \times 10^3$	15
4	$2.12 \pm 0.48 \times 10^3$	19
5	$2.66 \pm 0.71 \times 10^3$	22
6	$2.55 \pm 0.44 \times 10^3$	27
7	$2.22 \pm 0.55 \times 10^3$	33
14	$1.22 \pm 0.73 \times 10^2$	87
28	$1.73 \pm 0.22 \times 10^2$	93

After the inoculation of viable bacteria 10^4 cfu/g in chicken meat sample, the culture was kept at 4°C. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

Table 18. Chemotactic effects of 10% mucin-bile constituents on the numbers of starved *Campylobacter* isolated from artificially-inoculated chicken samples.

Campylobacter	Numbers of viable cells (Mean ± SE)	
	37°C	42°C
C. jejuni ATCC 33291	$2.28 \pm 0.23 \times 10^4$	$2.03 \pm 0.47 \times 10^5$
C. coli MUMT 18407	$2.34 \pm 0.33 \times 10^4$	$2.23 \pm 0.35 \times 10^5$
C. lari ATCC 43675	$2.21 \pm 0.25 \times 10^5$	$3.29 \pm 0.52 \times 10^6$
C. upsaliensis DMST 19055	$2.45 \pm 0.91 \times 10^4$	$2.31 \pm 0.24 \times 10^5$
Non-starved cells	$2.33 \pm 0.53 \times 10^8$	$1.78 \pm 0.38 \times 10^9$

After the inoculation of viable bacteria 10^4 cfu/g in chicken meat sample, the culture was kept at 4°C for 28 days. Viable counts on modified charcoal cefoperazone desoxycholate agar, incubated under microaerobic conditions at 42°C for 24 h. All experiments were carried out twice and measurements were performed in triplicate.

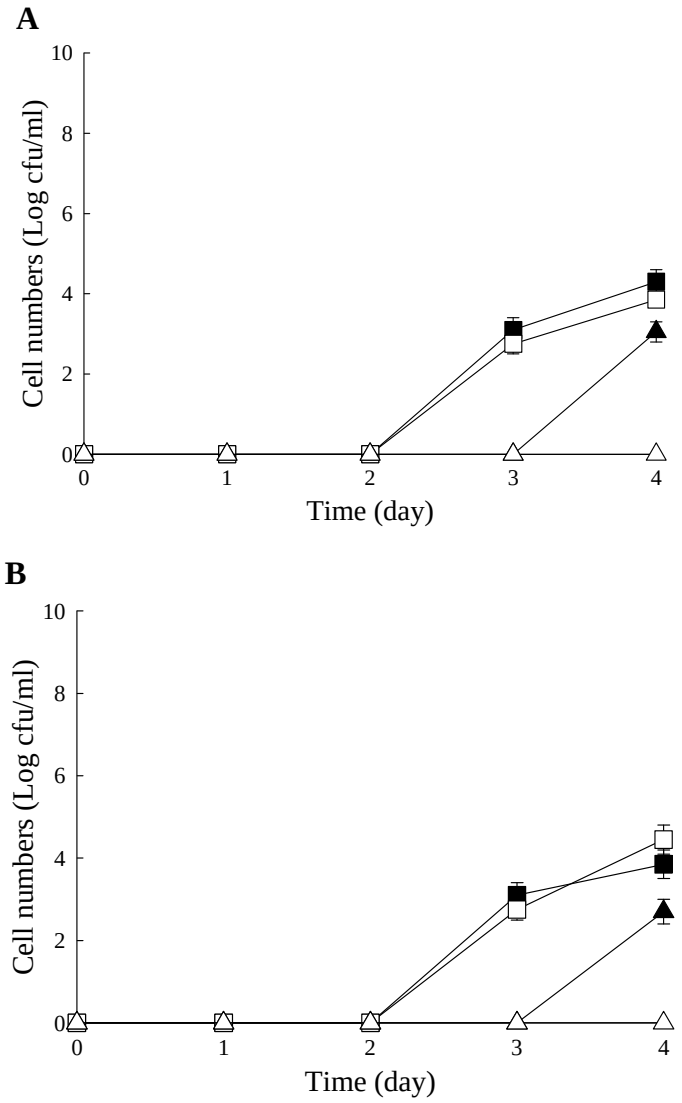

Figure 18. The recovery of *Campylobacter concisus* GC 99 (A) and *Helicobacter pylori* GC 11 (B) in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation into the enrichment chamber, the apparatus was incubated at 37°C. Viable counts were determined on Columbia blood agar with 15% defibrinated horse blood agar. The plates were incubated at 37°C for 24 h under microaerobic conditions.

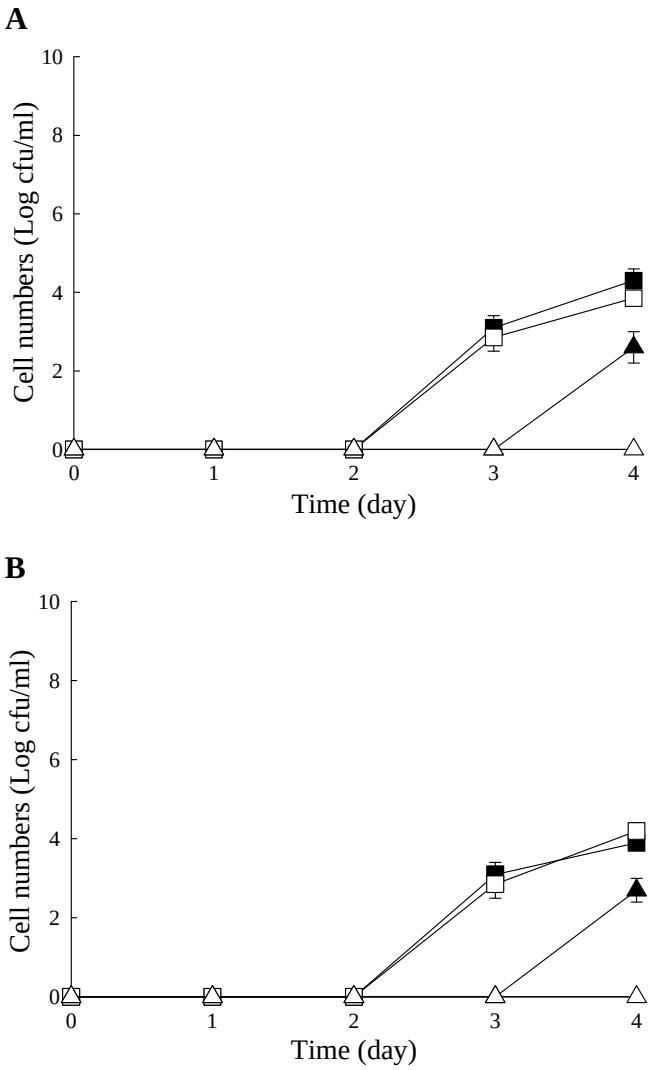

Figure 19. The recovery of *Helicobacter pylori* GC 17 (A) and *Helicobacter pylori* GC 51 (B) in *Campylobacter* apparatus. The initial bacterial concentration of each inoculum was 10^4 (■), 10^3 (□), 10^2 (▲), and 10 (Δ) cfu/ml. After the inoculation into the enrichment chamber, the apparatus was incubated at 37°C. Viable counts were determined on Columbia blood agar with 15% defibrinated horse blood agar. The plates were incubated at 37°C for 24 h under microaerobic conditions.

CHAPTER 5

DISCUSSION AND CONCLUSION

It is very difficult to detect small numbers of *Campylobacter* among large numbers of background microbiota in a complex sample matrix. Conventional cultural techniques used for detection of *Campylobacter* in food samples are time-consuming and laborious. Molecular methods based on PCR amplification are reliable alternatives for the detection of *Campylobacter*. A number of PCR-based methods have been reported for the detection of *Campylobacter* in food (Giesendorf *et al*., 1992; Oyofo *et al*., 1997; Denis *et al*., 1999; Waage *et al*., 1999; O'Sullivan *et al*., 2000; Grennan *et al*., 2001). However, these methods detect 10^3-10^4 cfu/g of target pathogens, meaning that pre-enrichment and enrichment steps are still necessary. Moreover, these methods are expensive and therefore not a feasible option for use in developing countries. Membrane filtration is physical methods that are commonly used to separate microorganisms from a complex sample matrix. However, it has been shown to require large numbers of organisms to allow detection (Goossens *et al*., 1990; Kulkarni *et al*., 2002). A combination of a selective enrichment procedure and membrane filtration was used to develop a novel apparatus for the detection of thermotolerant *Campylobacter* including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*.

Bolton broth supplemented with antibiotics was employed for the enrichment broth of thermophilic *Campylobacter*. It is recommended in protocols produced by the US Food and Drug Administration (US FDA, 2001) and The International Organization for Standardization (ISO, 2006) for the recovery of *Campylobacter* from food. Baylis *et al*. (2000) concluded that this broth represents the best overall compromise between growth of *Campylobacter* and inhibition of contaminants for the analysis of food samples. For most types of samples, the pre-enrichment period consists of incubation at 37°C for 4 h. Following enrichment, broths are transferred to 42°C for 24 h and 48 h (Humphrey, 1989; Bolton, 2000). It is recommended that resuscitation should be limited to 4 h to prevent overgrowth by contaminants (Goosens and Bultzer, 1992). In generally, Bolton broth contains blood to quench toxic oxygen compounds (Bolton *et al*., 1984).

However, blood-free Bolton broth was suitable for growth of *C. jejuni* (Tran, 1998). Moreover, it was as effective as blood containing Bolton broth for isolating *Campylobacter* from food (Tran, 1998; Bolton *et al*., 2002; Murinda *et al*., 2004). Therefore, blood-free Bolton broth was used as an enrichment broth in this study. Incubation at 42^{o}C serves as an additional selectivity criterium for thermotolerant *Campylobacter*. Our results as have been previously demonstrated, which confirmed that the optimal growth temperature of *Campylobacter* was 42^{o}C (Newell, 2001; van Vliet and Ketley, 2001; Snelling *et al*., 2005). The results further confirmed that this apparatus was able to eliminate competing bacteria and promote the growth of *Campylobacter* at 42^{o}C.

Campylobacter are recognized as the highly motile bacteria whose corkscrew-like motility results in a very remarkable swimming behavior. Ferrero and Lee (1988) investigated that the motility of *C. jejuni* in solutions of varying viscosity was compared with other rod-shaped bacteria. Only *Campylobacter* were actively motile in highly viscous solutions with velocities ranging from 60-100 µm/s. Li *et al*. (2000) demonstrated that spirochetes can swim in a highly viscous, gel-like medium, such as that found in connective tissue. The spiral shape of *Campylobacter* like that of spirochaetes may strongly influence its motile ability in Bolton broth containing 0.5% agar coated filter that inhibits the motility of most other bacteria. The results indicated that the final optimized protocol consisted of 0.5% agar-coated filter in Bolton broth at 42^{o}C for 24 h.

The *Campylobacter* apparatus exhibits high sensitivity. This device produced a detectable signal with a minimum bacterial density of 10 cfu/g. Since a total volume of 100 ml of the enrichment broth was in the chamber, the minimal bacterial density therefore corresponded to 0.1 cfu/ml. In previous studies detected very low levels of *Campylobacter* in food samples. Yang *et al*. (2003) found a sensitivity of 6 to 15 cfu per PCR in a real-time assay for detecting *C. jejuni* in poultry, milk, and environmental water, while a sensitivity of 10 cfu/ml was detected in a SYBR Green real-time PCR assay (Cheng *et al*., 2003). It was decided that filtration should be performed first to select bacteria that could pass though the agar-coated filter, and that post-enrichment would allow the multiplication of target bacteria, thereby giving high sensitivity even with a low number of *Campylobacter* cells in the samples.

Although *C. jejuni* is the only *Campylobacter* species that hydrolyses hippurate, analysis of the data revealed that results from biochemical identification were not in agreement with multiplex PCR. Most isolates gave the same results by both methods, except 3 (3.85%) strains that initially identified as *C. coli* by the biochemical

were confirmed to be *C. jejuni* by PCR technique. These strains are believed to represent a *C. jejuni* strain not expressing hippurate hydrolysis activity *in vitro*. I expected a difference due to the absence of hippurate hydrolysis for some *C. jejuni* as it has been generally observed (Totten *et al.,* 1987; Nicholson and Patton, 1995; Steinhauserova *et al.,* 2001; Waino *et al.,* 2003; Kos *et al.,* 2006). Moreover, 5 of 78 strains (6.41%) demonstrated positive hippurate test turned out to be mixed culture of *C. jejuni* and *C. coli*. This difference may due to positive hippurate test for isolates identified as *C. coli* by our multiplex PCR (3 isolates; 3.85%). False-positive hippurate test has been reported by other studies (Nicholson and Patton, 1995; Denis *et al.,* 1999; Waino *et al.,* 2003). Recently, Nakari *et al.* (2008) demonstrated that standardizing the hippurate test by determining minimum and maximum turbidity limits (McFarland 6 and McFarland 10) for the bacterial cell suspension eliminated the false positive results.

Recent studies analysing the distribution of separate *cdtA*, *cdtB*, *cdtC* genes or *cdt* clusters in *C. jejuni* and *C. coli* indicate their prevalence in isolates from poultry and other sources exceeds 90% (Bang *et al.*, 2001; Datta *et al.,* 2003; Wardak and Szych, 2006; Talukder *et al.,* 2008). In particular, *cdtB* was reported to be widely distributed in *C. jejuni* and *C. coli* strains by PCR (Pickett *et al.*, 1996; Eyigor *et al.,* 1999; Bang *et al.*, 2001; Bang *et al.,* 2003; Dassanayake *et al.,* 2005). Pickett *et al*. (1996) showed that *cdtB* gene was also found in *C. lari* and *C. upsaliensis*. The results confirmed high prevalence of *cdtB* gene in *C. jejuni* isolates from both clinical and chicken caeca. Similar observations indicating that the *cdtB* gene is present in *Campylobacter* isolated from human specimens as well as chicken carcasses have been reported by other authors. *cdtB* gene is an important virulence factor for *Campylobacter*. It was demonstrated that *cdtB* gene was found in 97.6% *C. jejuni* isolates, whereas no PCR product was detected for *C. coli* isolates (Zhang *et al.,* 2007). Similarly, *cdtB* was detected in 88% and 14% C. *jejuni* and *C. coli* isolates, respectively (Jain *et al.*, 2008).

It has been shown that *C. jejuni* was chemotactically affected by some carbohydrates, amino acids, sodium salts, and mucin-bile constituents. L-fucose was the only carbohydrate and, L-cysteine, L-glutamate, L-aspartate, and L-serine were the amino acids producing a chemotactic response. Several salts of organic acids, including pyruvate, succinate, fumarate, citrate, malate, and α-ketoglutarate, were also chemoattractants, as were mucin-bile constituents (Hugdahl *et al.,* 1988). In addition, *C. jejuni* was also attracted towards cysteine, phenylalanine and L-tryptophan (Khanna *et al.,* 2006). In this

study, viable cells of thermophilic *Campylobacter* were chemotactic toward mucin-bile constituents, but not to other compound tested.

The gastrointestinal tract is lined by continuously secreted mucus layer. Mucin is complex glycoproteins that comprises the major component of mucus and give mucus its viscous consistency (Hugdahl *et al.,* 1988). Mucin has been demonstrated to be a chemoattractant for *C. jejuni* (Hugdahl *et al.,* 1988). This component may carry physiologically relevant oligosaccharide ligands for the adhesion of *Campylobacter*. Most of the glycoprotein in gall-bladder bile is soluble mucus (Pearson *et al.,* 1982). Mucin and its constituent, L-fucose, are present in mucus and were the two chemoattractants identified in bile. Bile was composed of bile salts, bile pigments, cholesterol, phospholipids, and proteins. It was synthesized in the liver from cholesterol and bile acids (Rhoades and Tanner, 1995). Bile attracts *C. jejuni* despite the repellent effect of the bile acids (Hugdahl *et al.,* 1988). Thus, the soluble mucin in bile is a significant factor in the attraction of *C. jejuni* to bile. L-fucose has been suggested to be a key element required in intestinal colonization of *Campylobacter* (Lee *et al.,* 1986; Hugdahl *et al.,* 1988). The concentration of fucose in mucin ranges from 10-11.3% (Scawen and Allen, 1977; Glenister *et al*., 1988). Therefore, 10% mucin-bile constituents were selected for this study.

We hypothesize that substrates contained in intestinal mucus may constitute a source of energy. Mucin 2 (MUC2) is the main secreted mucin making up the mucus layer in human intestine. Tu *et al*. (2008) demonstrated that *C. jejuni* utilizes MUC2 for the modulation of expression of virulence genes including cytolethal distending toxin protein, *Campylobacter* invasion antigen, putative mucin-degrading enzymes, and flagellin A proteins. Furthermore, the results of this study showed that the optimal temperature for chemotaxis of *Campylobacter* to mucin-bile constituents was 42^{o}C, which corresponds to the optimal growth temperature for the bacterium.

The flagellum of *Campylobacter* consists of two flagellin proteins including FlaA and FlaB (Alm *et al.,* 1993). The *flaA* and *flaB* genes are transcribed from different promoters. A classical *c28* flagellin promoter controls transcription of *flaA*. Whereas, *flaB* is regurated by *c54* promoter (Nuijten *et al.,* 1990; Guerry *et al.,* 1991; Hendrixson and DiRita, 2003). The motility of *C. jejuni* increases in highly viscous solutions (Lee *et al*., 1986; Ferrero and Lee, 1988). Allen and Griffiths (2001) also showed that bovine bile, deoxycholate, and L-fucose up-regulated the *flaA* promoter while viscosity resulted in down-regulation.

Change in the morphological structure of *Campylobacter* from spiral to coccoid has been observed during environmental stress (Ng *et al.*, 1985). The coccoid forms tend to be difficult to subculture and lose motility (Oliver, 2005). In this study at 4^{o}C, loss of culturability of *C. jejuni* occurred dependent of the formation of coccoid cells. In addition, starved *Campylobacter* still moved through the soft-agar-coated filter, but reduced chemotactic response towards mucin-bile constituents. Data from other study showed that starved *Vibrio anguillarum* cells decreased chemotactic response towards fish mucus and serine when exposed to low temperatures and high salinity (Larsen *et al.*, 2004). In conclusion, the present studies indicate that mucin-bile constituents could reduce incubation time in this apparatus. However, this system could not be affected on thermophilic *Campylobacter* under long-term storage at low temperature.

Nevertheless, using this new culture method and using the multiplex PCR identifies the species of *Campylobacter* to determine the prevalence of thermophilic *Campylobacter* species in chicken meat products demonstrated that *Campylobacter* were detected in 14.75%. Of the 305 samples, *Campylobacter* were detected in 15.33% and 14.19% of fresh chicken samples and processed chicken samples, respectively. In processed chicken samples, 20.95% frozen chicken samples were positive with *Campylobacter*, whereas no *Campylobacter* were isolated from ready-to-eat chicken samples. The smaller percentages of contamination in chicken samples suggested that fresh and processed chicken samples could be hygienically processed from the slaughterhouse to the market throughout. *C. coli* was the most common species detected in this study. Similar observations indicating that *C. coli* was the dominant *Campylobacter* isolated from chicken in Thailand have been reported (Meeyam *et al*., 2004; Padungtod and Kaneene, 2005). In adition, *cdtB* gene was present in 100% (3/3) of *C. jejuni* isolated. Contrastly, this gene was not found in *C. coli* isolated.

Helicobacter pylori is a common human pathogen and public health problem that causes gastritis and peptic ulcers (Azevedo *et al*., 2009; Costa *et al*., 2009). Recently, Zhang *et al*. (2009) presented data on the isolation of *C. concisus* from gastric biopsy specimens with inflammatory bowel diseases associated. Detection method of *H. pylori* and *C. concisus* is usually based on endoscopy followed by bacterial culture of gastric biopsy sections (Sainsus *et al*., 2008). However, conventional cultural methods are cumbersome, time-consuming, and require a special atmosphere-generating system. The rapid urease reaction can be used as an indicator test for the presence of urease-positive *H. pylori* in the culture. However, the presence of urea in culture media may

negatively affect *H. pylori* viability (Clyne *et al*., 1995). Preliminary study showed that *H. pylori* and *C. concisus* moved through the membrane filter and could multiply in the enrichment culture. Interestingly, the results demonstrated that this apparatus may be used for direct isolations from gastric biopsies. Unfortunately, all samples were negative for *C. concisus* and *H. pylori*. However, a larger sample size is required for the validation of this application.

In summary, this apparatus demonstrates useful information on an innovative new method for the isolation of viable thermophilic *Campylobacter* species. The advantages of *Campylobacter* apparatus are (i) the ability of separating viable from dead cells; (ii) prevention of food matrix particles; (iii) maintenance and promote of cell viability. Besides, it is cost-effective and can be extended to other environmental samples. A multiplex PCR allows in a single tube PCR, the identification of the four clinically important *Campylobacter* species, with a simultaneous detection of the *cdtB* gene. The PCR works well in our hands with both purified genomic DNA and whole-cell suspension. This should greatly speed up identification by replacing the current biochemical phenotypic schemes. In addition, the system can detect the presence of *cdtB* gene which encodes the cytolethal-distending toxin activity.

REFERENCES

Aarestrup, F.M., Nelsen, E.M., Madsen, M. and Engberg, J. 1997 Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from humans, pigs, cattle, and broilers in Denmark. Antimicrob. Agents Chemother. 41: 2244-2250.

Aarestrup, F.M. and Wegener, H.C. 1999 The effects of antibiotic usage in food animals on the development of antimicrobial resistance for humans in *Campylobacter* and *Escherichia coli*. Microbes Infect. 1: 639-644.

Abeyta, C.Jr., Trost, P., Bark, D.H., Hunt, J.M., Kaysner, B.J., Tenge, C. and Wekell, M.M. 1997 The use of bacterial membrane fractions for the detection of *Campylobacter* species in shellfish. J Rapid Methods Automation Microbiol. 5: 223-247.

Adam, M., Contzen, M., Horlacher, S. and Rau, J. 2006 Prevalence of *Campylobacter* spp. in poultry meat and raw milk using PCR, conventional cultural methods and Fourier transform infrared spectroscopy. Berl. Munch. Tierarztl. Wochenschr. 119: 209-215.

Adler, H.I. and Crow, W.D. 1981 A novel approach to the growth of anaerobic microorganisms. Biotechnol Bioeng Symp. 11: 533-540.

Allen, K.J. and Griffiths, M.W. 2001 Effect of environmental and chemotactic stimuli on the activity of the *Campylobacter jejuni flaA* sigma (28) promoter. FEMS Microbiol. Lett. 205: 43-48.

Allos, B.M. 1997 Association between *Campylobacter* infection and Guillain-Barre syndrome. J. Infect. Dis. 176: 125-128.

Allos, B.M. 2001 *Campylobacter jejuni* Infections: update on emerging issues and trends. Clin. Infect. Dis. 32: 1201-1206.

Altekruse, S.F., Stern, N.J., Fields, P.I. and Swerdlow, D.L. 1999 *Campylobacter jejuni* an emerging foodborne pathogen. Emerging Infect. Dis. 5: 28-35.

Alm, R.A., Guerry, P. and Trust, T.J. 1993 Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni*. J. Bacteriol. 175: 3051-3057.

Asakura, M., Samosornsuk, W., Taguchi, M., Kobayashi, K., Misawa, N., Kusumoto, M., Nishimura, K., Matsuhisa, A. and Yamasaki, S. 2007 Comparative analysis of cytolethal distending toxin (cdt) genes among *Campylobacter jejuni*, *C. coli* and *C. fetus* strains. Microb. Pathog. 42: 174-183.

Axelsson-Olsson, D., Waldenstrom, J., Broman, T., Olsen, B. and Holmberg, M. 2005 Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. Appl. Environ. Microbiol. 71: 987-992.

Azevedo, N.F., Huntington, J. and Goodman, K.J. 2009 The epidemiology of *Helicobacter pylori* and public health implications. Helicobacter. 14 1: 1-7.

Bacon, D.J., Alm, R.A., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., Trust, T.J. and Guerry, P. 2000 Involvement of a plasmid in virulence of *Campylobacter jejuni* 81176. Infect. Immun. 68: 4384-4390

Bacon, D.J., Alm, R.A., Hu, L., Hickey, T.E., Ewing, C.P., Batchelor, R.A., Trust, T.J. and Guerry, P. 2002 DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81176. Infect. Immun. 70: 6242-6250.

Bang, D.D., Scheutz, F., Ahrenz, P., Pedersen, K., Blom, J. and Madsen, M. 2001 Prevalence of cytolethal distending toxin (*cdt*) genes and CDT production in *Campylobacter* spp. isolated from Danish broilers. J. Med. Microbiol. 50: 1087-1094.

Bang, D.D., Wedderkopp, A., Pedersen, K. and Madsen, M. 2002 Rapid PCR using nested primers of the 16S rRNA and the hippuricase (hip O) genes to detect

Campylobacter jejuni and *Campylobacter coli* in environmental samples. Mol. Cell. Probes. 16: 359-369.

Bang, D.D., Nielsen, E.M., Scheutz, F., Pedersen, K., Handberg, K. and Madsen, M. 2003 PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. J. Appl. Microbiol. 94: 1003-1014.

Baylis, C.L., MacPhee, S., Martin, K.W., Humphrey, T.J. and Betts, R.P. 2000 Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. J. Appl. Microbiol. 89: 884-891.

Biswas, D., Itoh, K. and Sasakawa, C. 2000 Uptake pathways of clinical and healthy animal isolates of *Campylobacter jejuni* into INT-407 cells. FEMS Immunol. Med. Microbiol. 29: 203-211.

Black, R.E., Levine, M.M., Clements, M., Hughes, T.P. and Blaser, M.J. 1988 Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. 157: 472-479.

Bodhidatta, L., Vithayasai, N., Eimpokalarp, B., Pitarangsi, C., Serichantalergs, O. and Isenbarger, D.W. 2002 Bacterial enteric pathogens in children with acute dysentery in Thailand: increasing importance of quinolone-resistant *Campylobacter*. Southeast Asian J. Trop. Med. Public Health. 33: 752-757.

Bodhidatta, L., Lan, N.T., Hien, B.T., Lai, N.V., Srijan, A., Serichantalergs, O., Fukuda, C.D., Cam, P.D. and Mason, C.J. 2007 Rotavirus disease in young children from Hanoi, Vietnam. Pediatr. Infect. Dis. J. 26: 325-328.

Bolton, F.J. and Robertson, L. 1982 A selective medium for isolating *Campylobacter jejuni*/*coli*. J. Clin. Pathol. 35: 462-467.

Bolton, D. Coates, D. N. Hutchinson, A.F. and Godfree, F.J. 1984 Hutchinson and D. Coates, A blood-free selective medium for the isolation of *Campylobacter jejuni* from faeces. J. Clin. Microbiol. 19: 169-171.

Bolton, F.J., Coates, D. and Hutchinson, D.N. 1984 The ability of *Campylobacter* supplements to neutralize photochemically induced toxicity and hydrogen peroxide. J. Appl. Bacteriol. 56: 151-157.

Bolton, F. 2000 Methods for isolation of *Campylobacters* from humans, food and water. *In*: The increasing incidence of human campylobacteriosis. Report and Proceedings of a WHO Consultation of Experts, Copenhagen, Denmark 21-25 November 2000, p87-93.

Bolton, F.J., Sails, A.D., Fox, A.J., Wareing, D.R. and Greenway, D.L., 2002 Detection of *Campylobacter jejuni* and *Campylobacter coli* in foods by enrichment culture and polymerase chain reaction enzyme-linked immunosorbent assay. J. Food Prot. 65: 760-767.

Bourke, B. 2002 *Campylobacter* infection: small bowel and colon. Curr. Opin. Gastroenterol. 18: 4-9.

Bovill, R.A. and Mackey, B.M. 1997 Resuscitation of 'non-culturable' cells from aged cultures of *Campylobacter jejuni*. Microbiology. 143: 1575-1581.

Brouwer, R., Mertens, M.J., Siem, T.H. and Katchaki, J. 1979 An explosive outbreak of *Campylobacter* enteritis in soldiers. Antonie Van Leeuwenhoek. 45: 517-519.

Burnett, T.A., Hornitzky, M.A., Kuhnert, P. and Djordjevic, S.P. 2002 Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays. FEMS Microbiol. Lett. 216: 201-209.

Cappelier, J.M., Magras, C., Jouve, J.L. and Federighi, M. 1999 Recovery of viable but non-culturable *Campylobacter jejuni* cells in two animal models. Food Microbiol. 16: 375-383.

Cardinale, E., Tall, F., Guèye, E.F., Cisse, M. and Salvat, G. 2004 Risk factors for *Campylobacter* spp. infection in Senegalese broiler-chicken flocks. Prev. Vet. Med. 64: 15-25.

CDC, Centers for Disease Control and Prevention. 2000 Preliminary FoodNet data on the incidence of foodborne illnesses selected sites, United States, 1999. 49: 201-205.

CDC, Centers for Disease Control and Prevention. 2007 Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food 10 States, United States, 2008. 56: 366-370.

CDC, Centers for Disease Control and Prevention. 2008 Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food 10 States, 2008. 58: 333-337.

Chan, A.B. and Fox, J.D. 1999 NASBA and other transcription-based amplification methods for research and diagnostic microbiology. Rev. Med. Microbiol. 10: 185-196.

Charvalos, E., Tselentis, Y., Hamzehpour, M.M., Kohler, T. and Pechere, J.C. 1995 Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrob. Agents Chemother. 39: 2019-2022.

Cheng, Z. and Griffiths, M.W. 2003 Rapid detection of *Campylobacter jejuni* in chicken rinse water by melting-peak analysis of amplicons in real-time polymerase chain reaction. J. Food Prot. 66: 1343-1352.

Clyne, M., Labigne, A. and Drumm, B. 1995 *Helicobacter pylori* requires an acidic environment to survive in the presence of urea. Infect. Immun. 63: 1669-1673.

Cools, I., Uyttendaele, M., Caro, C., D'Haese, E., Nelis, H.J. and Debevere, J. 2003 Survival of *Campylobacter jejuni* strains of different origin in drinking water. J. Appl. Microbiol. 94: 886-892.

Corcoran, D., Quinn, T., Cotter, L., O'Halloran, F. and Fanning, S. 2005 Characterisation of a cmeABC operon in a quinolone-resistant *Campylobacter coli* isolate of Irish origin. Microb. Drug Resist. 11: 303-308.

Corry, J.E., Post, D.E., Colin, P. and Laisney, M.J. 1995 Culture media for the isolation of *Campylobacter*. Int. J. Food Microbiol. 26: 43-76.

Costa, A.C., Figueiredo, C. and Touati, E. 2009 Pathogenesis of *Helicobacter pylori* infection. Helicobacter. 14: 15-20.

Crushell, E., Harty, S., Sharif, F. and Bourke, B. 2004 Enteric *Campylobacter*: purging its secrets. Pediatr. Res. 55: 3-12.

Cui, S., Ge, B., Zheng, J. and Meng, J. 2005 Prevalence and antimicrobial resistance of Campylobacter spp. and Salmonella serovars in organic chickens from Maryland retail stores. Appl. Environ. Microbiol. 71: 4108-4111.

Dassanayake, R.P., Zhou, Y., Hinkley, S., Stryker, C.J., Plauche, G., Borda, J.T., Sestak, K. and Duhamel, G.E. 2005 Characterization of cytolethal distending toxin of *Campylobacter* species isolated from captive macaque monkeys. J. Clin. Microbiol. 43: 641-649.

Datta, S., Niwa, H. and Itoh, K. 2003 Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. J. Med. Microbiol. 52: 345-348.

Denis, M., Soumet, C., Rivoal, K., Ermel, G., Blivet, D., Salva, G. and Colin, P., 1999 Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett. Appl. Microbiol. 29: 406-410.

DLD, Department of Livestock Development. 2007 Livestock Statistics. Royal Thai Government. [Available from: www.dld.go.th].

DLD, Department of Livestock Development. 2008 Livestock Statistics. Royal Thai Government. [Available from: www.dld.go.th].

Doyle, M.P. and Jones, D.M. 1992 Food-borne transmission and antibiotic resistance of *Campylobacter jejuni*. *In*: *Campylobacter jejuni*: Current status and future trends. Ed I. Nachamkin, M.J. Blaser and L.S. Tompkins. ASM, Washington, USA. p45-48.

Draghici, S. 2003 Microarrays. In: Data analysis tools for DNA microarrays. Chapman and Hall/CRC Press, Boca Raton.

Englen, M.D., Ladely, S.R. and Fedorka-Cray, P.J. 2003 Isolation of *Campylobacter* and identification by PCR. Methods Mol. Biol. 216: 109-121.

ESR, Institute of Environmental Science ad Research Limited. Annual report 2006 Notifiable and other diseases in New Zealand.

European Food Safety Authority. 2007 The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006, EFSA. 130: 1-310.

European Food Safety Authority. 2009 The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006, EFSA. 13: 1-310.

Eyers, M., Chapelle, S., Van Camp, G., Goossens, H. and De Wachter, R. 1993 Discrimination among thermophilic *Campylobacter* species by polymerase chain reaction amplification of 23S rRNA gene fragments. J. Clin. Microbiol. 31: 3340-3343.

Eyigor, A., Dawson, K.A., Langlois, B.E. and Pickett, C.L. 1999 Detection of cytolethal distending toxin activity and *cdt* genes in *Campylobacter* spp. isolated from chicken carcasses. Appl. Environ. Microbiol. 65: 1501-1505.

Fearnley, C., Ayling, R.D. and Newell, D.G. 1994 The failure of viable but non-culturable *Campylobacter jejuni* to colonise the chick model. Report of a WHO consultation on epidemiology and control of campylobacteriosis in animals and humans. World Health Organization, Geneva, Switzerland. p85-87.

Ferrero, R.L. and Lee, A. 1988 Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. J. Gen. Microbiol. 134: 53-59.

Friedman, C.R., Neimann, J., Wegener, H.C. and Tauxe, R.V. 2000 Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialised nations. In: Nachamkin, I., Blaser, M.J. (Eds.), *Campylobacter*. American Society for Microbiology, Washington, DC, p121-138.

FSIS USDA, Food Safety and Inspection Service/U.S. Department of Agriculture. 2001. Available at: http://www.fsis.usda.gov/Science/Baseline_Data/index.asp. Accessed 2007Oct 17

Gaunt, P.N. and Piddock, L.J. 1996 Ciprofloxacin resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. J. Antimicrob. Chemother. 37: 747-757.

George, H.A., Hoffman, P.S., Smibert, R.M. and Krieg, N.R. 1978 Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. 8: 36-41.

Gibreel, A., Sjogren, E., Kaijser, B., Wretlind, B. and Skold, O. 1998 Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational chages in gyrA and parC. Antimicrob. Agents Chemother. 42: 3276-3278.

Giesendorf, B.A., W.G. Quint, M.H. Henkens, H. Stegeman, Huf, F.A. and Niesters, H. G. 1992 Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3804-3808.

Glenister, A., Salamon, K.E., Smith, K., Beighton, D. and Keevil, C.W. 1988 Enhanced growth of complex communities of dental plaque bacteria in mucin-limited continuous culture. Microb. Ecol. Health Dis. 1: 31-38.

Gonzalez, I., Grant, K.A., Richardson, P.T., Park, S.F. and Collins, M.D. 1997 Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceuE* gene encoding a putative virulence determinant. J. Clin. Microbiol. 35: 759-763.

Goossens, H., Pot, B., Vlaes, L., Van den Borre, C., Van den Abbeele, R., Van Naelten, C., Levy, J., Cogniau, H., Marbehant, P., Verhoef, J., Kersters, K., Butzler, J. and Vandamme, P. 1990 Characterization and description of "*Campylobacter upsaliensis*" isolated from human feces. J. Clin. Microbiol. 28: 1039-1046.

Goossens, H., Vlaes, L., Butzler, J.P., Adnet, A., Hanicq, P., N'jufom, S., Massart, D., Schrijver, G.D. and Blomme, W. 1991 *Campylobacter upsaliensis* enteritis associated with canine infections. Lancet. 337: 1486-1487.

Goosens, H. and Butzler, J.P. 1992 Isolation and identification of *Campylobacte*r spp. *In*: *Campylobacter jejuni*: Current status and future trends. Ed I. Nachamkin, M.J. Blaser and L.S. Tompkins. ASM, Washington, USA. p93-109.

Grennan, B., O'Sullivan, N.A., Fallon, R., Carroll, C., Smith, T., Glennon, M. and Maher, M. 2001 PCR-ELISAs for the detection of *Campylobacter jejuni* and *Campylobacter coli* in poultry samples. BioTechniques. 30: 602-606.

Guerry, P., Alm, R.A., Power, M.E., Logan, S.M. and Trust, T.J. 1991 Role of two flagellin genes in *Campylobacter* motility. J. Bacteriol. 173, 4757-4764.

Guerry, P., Alm, R.A., Power, M.E. and Trust, T.J. 1992 Molecular and structural analysis of *Campylobacter* flagellin. In *Campylobacter jejuni*: Current status and future trends. I. Nachamkin, M. J. Blaser, and L. S. Tompkins (Ed.). American Society for Microbiology, Washington, D.C.

Hald, B., Knudsen, K., Lind, P. and Madsen, M. 2001 Study of the infectivity of saline-stored *Campylobacter jejuni* for day-old chicks. Appl. Environ. Microbiol. 67: 2388-2392.

Hawkey, PM. 2003 Mechanisms of quinolone action and microbial response. J. Antimicrob. Chemother. 51: 29-35.

Hayashi, S., Okura, M. and Osawa, R. 2006 Soft-agar-coated filter method for early detection of viable and thermostable direct hemolysin (TDH)- or TDH-related hemolysin-producing *Vibrio parahaemolyticus* in seafood. Appl. Environ. Microbiol. 72: 4576-4582.

Hazeleger, W.C., Janse, J.D., Koenraad, P.M., Beumer, R.R., Rombouts, F.M. and Abee, T. 1995 Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. Appl. Environ. Microbiol. 61: 2713-2719.

Hendrixson, D.R. and DiRita, V.J. 2003 Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. Mol. Microbiol. 50: 687-702.

Hickey, T.E., Baqar, S., Bourgeois, A.L., Ewing, C.P. and Guerry, P. 1999 *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. Infect. Immun. 67: 88-93.

Hoge, C.W., Gambel, J.M., Srijan, A., Pitarangsi, C. and Echeverria, P. 1998 Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clin. Infect. Dis. 26: 341-345.

Hooper, D.C. 2000 New uses for new and old quinolones and the challenge of resistance. Clin. Infect. Dis. 30: 243-254.

Houng, H.S., Sethabutr, O., Nirdnoy, W., Katz, D.E. and Pang, L.W. 2001 Development of a *ceuE*-based multiplex polymerase chain reaction (PCR) assay for direct

detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in Thailand. Diagn. Microbiol. Infect. Dis. 40: 11-19.

Hu, L. and Kopecko, D.J. 1999 *Campylobacter jejuni* 81–176 associates with microtubules and dynein during invasion of human intestinal cells. Infect. Immun. 67: 4171-4182.

Hugdahl, M.B., Beery, J.T. and Doyle, M.P. 1988 Chemotactic behavior of *Campylobacter jejuni*. Infect. Immun. 56: 1560-1566.

Humphrey, T.J. and Cruikshank, J.G. 1985 Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. J. Appl. Bacteriol. 59: 65-71.

Humphrey, T.J. 1989 An appraisal of the efficacy of pre-enrichment for the isolation of *Campylobacter jejuni* from water and food. J. Appl. Bacteriol. 66: 119-126.

Humphrey, T.J. 1990 The synergistic inhibition of *Campylobacter jejuni* by rifampicin and hydrogen peroxide. Lett. Appl. Microbiol. 10: 97-100.

Humphrey, T., O'brien, S. and Madsen, M. 2007 *Campylobacters* as zoonotic pathogens: A food production perspective. Int. J. Food Microbiol. 117: 237-257.

Hunt, J.M., Abeyta, C. and Tran, T. 1998 Bacteriological Analytical Manual, 8th Edition, Revision A, Chapter 7.

ISO, 2006 Microbiology of food and animal feeding stuffs-Horizontal method for detection and enumeration of *Campylobacter* spp. Detection method. International Standard ISO/10272-1.

Jacobs-Reitsma, W.F. and de Boer, E. 2001 Revision of ISO 10272:1995 – Detection of thermotolerant Campylobacter in foods. Presented to the 11th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Freiburg Germany, 1-5 September 2001, IJMM Volume 291, Supplement No. 31. L-01.

Jacoby, G.A., Chow, N. and Waites, K.B. 2003 Prevalence of plasmid-mediated quinolone resistance. Antimicrob. Agents. Chemother. 47: 559-562.

Jain, D., Prasad, K.N., Sinha, S. and Husain, N. 2008 Differences in virulence attributes between cytolethal distending toxin positive and negative *Campylobacter jejuni* strains. J. Med. Microbiol. 57: 267-272.

Jin, S., Joe, A., Lynett, J., Hani, E.K., Sherman, P. and Chan, V.L 2001 JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. 39: 1225-1236.

Johnson, W.M. and lior, H. 1988 A new heat-labile cytolethal distending tixin (CLDT) produced by *Campylobacter* spp. Microb. Pathog. 4: 115-126.

Jones, D.M., Sutcliffe, E.M. and Curry, A. 1991 Recovery of viable but non-culturable *Campylobacter jejuni*. J. Gen. Microbiol. 137: 2477-2482.

Jones, K., Howard, S. and Wallace J.S. 1999 Intermittent shedding of thermotolerant campylobacter by sheep at pasture. J. Appl. Microbiol. 86: 531-536.

Karlyshev, A.V., McCrossan, M.V., and Wren, B.W. 2001 Demonstration of polysaccharide capsule in *Campylobacter jejuni* using electron microscopy. Infect. Immun. 69: 5921-5924.

Katzav, M., Isohanni, P., Lund, M., Hakkinen, M. and Lyhs, U. 2008 PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. Food Microbiol. 25: 908-914.

Keer, J.T. and Birch, L. 2003 Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods. 53: 175-183.

Khanna, M.R., Bhavsar, S.P. and Kapadnis, B.P. 2006 Effect of temperature on growth and chemotactic behaviour of *Campylobacter jejuni*. Lett. Appl. Microbiol. 43: 84-90.

Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R. and Lens, P. 1991 NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Methods. 35: 273-286.

Klena, J.D., Parker, C.T., Knibb, K., Ibbitt, J.C., Devane, P.M., Horn, S.T., Miller, W.G. and Konkel, M.E. 2004 Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. J. Clin. Microbiol. 42: 5549-5557.

Konkel, M.E. and Joens, L.A. 1989 Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. 57: 2984-2990.

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson, D.E.Jr. and Cieplak, W.Jr. 1997 Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol. Microbiol. 24: 953-963.

Konkel, M.E., Kim, B.J., Rivera-Amill, V. and Garvis, S.G. 1999 Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells. Mol. Microbiol. 32: 691-701.

Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B. and Mickelson, J. 2004 Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. J. Bacteriol. 186: 3296-3303.

Kopecko, D.J., Hu, L. and Zaal, K.J. 2001 *Campylobacter jejuni* microtubule-dependent invasion. Trends. Microbiol. 9: 389-396.

Korhonen, L.K. and Martikainen, P.J. 1991 Survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water. J. Appl. Bacteriol. 71: 379-382.

Kos, V.N., Gibreel, A., Keelan, M. and Taylor, D.E. 2006 Species identification of erythromycin-resistant *Campylobacter* isolates and optimization of a duplex PCR for rapid detection. Res. Microbiol. 157: 503-507.

Kulkarni, S.P., Lever, S., Logan, J.M., Lawson, A.J., Stanley, J. and Shafi, M.S. 2002 Detection of campylobacter species: a comparison of culture and polymerase chain reaction based methods. J. Clin. Pathol. 55: 749-753.

Lamoureux, M., MacKay, A., Messier, S., Fliss, I., Blais, B.W., Holley, R.A. and Simard, R.E. 1997 Detection of *Campylobacter jejuni* in food and poultry viscera using immunomagnetic separation and microtitre hybridization. J. Appl. Microbiol. 83: 641-651.

Lara-Tejero, M. and Galán, J.E. 2001 CdtA, CdtB, and CdtC form a tripartate complex that is required for cytolethal distending activity. Infect. Immun. 69: 4358-4365.

Larsen, M.H., Blackburn, N., Larsen, J.L. and Olsen, J.E. 2004 Influences of temperature, salinity and starvation on the motility and chemotactic response of *Vibrio anguillarum*. Microbiology. 150: 1283-12890.

Lawson, A.J., Logan, J.M., O'neill, G.L., Desai, M. and Stanley, J. 1999 Large-scale survey of *Campylobacter* species in human gastroenteritis by PCR and PCR–enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37: 3860-3864.

Lee, A., O'Rourke, J.L., Barrington, P.J. and Trust, T.J. 1986 Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model. Infect. Immun. 51: 536-546.

Lastovica, A.J. and Skirrow, M.B. 2000 Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *C. coli*. *In*: Nachamkin, I.; Blaser, M.J. (eds.). *Campylobacter* 2nd Edition. ASM Press. Washington, DC, USA. p89-120.

Lawson, A.J., Logan, J.M., O'Neill, J., Desai, M. and Stanley, J. 1999 Large-scale survey of *Campylobacter* species in human gastroenteritis by PCR and PCR–enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37: 3860-3864.

Li, C., Motaleb, A., Sal, M., Goldstein, S.F. and Charon, N.W. 2000 Spirochete periplasmic flagella and motility. J. Mol. Microbiol. Biotechnol. 2: 345-354.

Lin, J., Michel, L.O. and Zhang, Q. 2002 CmeABC Functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob Agents Chemother. 46: 2134-2131.

Lin, J., Akiba, M., Sahin, O. and Zhang, Q. 2005 CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. Antimicrob. Agents Chemother. 49: 1067-1075.

Linton, D., Owen, R.J. and Stanley, J. 1996 Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. Res. Microbiol. 147: 707-718.

Linton, D., Lawson, A.J., Owen, R.J. and Stanley, J. 1997 PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. 35: 2568-2572.

Lior, H., Woodward, D.L., Edgar, J.A., Laroche, L.J. and Gill, P. 1982 Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J. Clin. Microbiol. 15: 761-768.

Little, C.L., Richardson, J.F., Owen, R.J., de Pinna, E. and Threlfall, E.J. 2008 Prevalence, characterisation and antimicrobial resistance of Campylobacter and Salmonella in raw poultry meat in the UK, 2003-2005. Int. J. Environ. Health Res. 18: 403-414.

Lukinmaa, S., Nakari, U.M., Eklund, M. and Siitonen, A. 2004 Application of molecular genetic methods in diagnostics and epidemiology of foodborne bacterial pathogens. APMIS. 112: 908-929.

Lund, M., Nordentoft, S., Pedersen, K. and Madsen, M. 2004 Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. J. Clin. Microbiol. 42: 5125-5132.

Luu, Q.H., Tran, T.H., Phung, D.C. and Nguyen, T.B. 2006 Study on the prevalence of Campylobacter spp. from chicken meat in Hanoi, Vietnam. Ann. N. Y. Acad. Sci. 1081: 273-275.

Maher, M., Finnegan, C., Collins, E., Ward, B., Carroll, C. and Cormican, M. 2003 Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of feces. J. Clin. Microbiol. 41: 2980-2986.

Martin, K.W., Mason, M.J., McAlpine, K.T. and Humphrey, T.J. 1996 A campylobacter medium for all seasons? *In: Campylobacters*, *Helicobacters* and related organisms eds. Newell, D.G., Ketley, J.M. and Feldman, R.A. Plenum Press, New York, p61-65.

Martin, K.W., Mattick, K.L., Harrison, M. and Humphrey, T.J. 2002 Evaluation of selective media for *Campylobacter* isolation when cycloheximide is replaced with amphotericin B. Lett. Appl. Microbiology. 34: 124-129.

Martinot, M., Jaulhac, B., Moog, R., De Martinoi, S., Kehrli, P., Monteil, H. and Piemont, Y. 2001 *Campylobacter lari* bacteremia. Clin. Microbiol. Infect. 7: 88-102.

Mateo, E., Cárcamo, J., Urquijo, M., Perales, I. and Fernández-Astorga, A. 2005 Evaluation of a PCR assay for the detection and identification of *Campylobacter jejuni* and *Campylobacter coli* in retail poultry products. Res. Microbiol .156: 568-574.

McKillip, J.L., Jaykus, L.A. and Drake, M.. 1998 rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157: H7. Appl. Environ. Microbiol. 64: 4264-4268.

Mdegela, R.H., Nonga, H.E., Ngowi, H.A. and Kazwala, R.R. 2006 Prevalence of thermophilic *Campylobacter* infections in humans, chickens and crows in Morogoro, Tanzania. J. Vet. Med. B Infect. Dis. Vet. Public Health. 53: 116-121.

Meeyam, T., Padungtod, P. and Kaneene, J.B. 2004 Molecular characterisation of *Campylobacter* isolated from chickens and humans in northern Thailand. Southeast Asian J. Trop. Med. Public Health. 35: 670-675.

Meijer, A., Roholl, P.J., Gielis-Proper, S.K. and Ossewaarde, J.M. 2000 *Chlamydia pneumoniae* antigens, rather than viable bacteria, persist in atherosclerotic lesions. J. Clin. Pathol. 53: 911-916.

Minihan, D., Whyte, P., O'Mahony, M., Cowley, D., O'Halloran, F., Fanning, S. and Collins, J.D. 2006 Phenotypic and genotypic anti-microbial resistance profiles of *Campylobacter* from untreated feedlot cattle and their environment. J. Vet. Med. 53: 181-187.

Ministry of Public Health. 2006 Annual Epidemiological Surveillance Report. Ministry of Public Health, Bangkok.

Moore, J.E., Corcoran, D., Dooley, J.S., Fanning, S., Lucey, B., Matsuda, M., McDowell, D.A., Megraud, F., Millar, B.C., O'Mahony, R., O'Riordan, L., O'Rourke, M., Rao, J.R., Rooney, P.J., Sails, A. and Whyte, P. 2005 *Campylobacter*. Vet. Res. 36: 351-382.

Moran, L., Kelly, C. and Madden, R.H. 2009 Factors affecting the recovery of Campylobacter spp. from retail packs of raw, fresh chicken using ISO 10272-1:2006. Lett. Appl. Microbiol. 48: 628-632.

Moreno, Y., Botella, S., Alonso, J.L., Ferrus, M.A., Hernandez, M. and Herhandez, J. 2003 Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. Appl. Environ. Microbiol. 69: 1181-1186.

Morris, G.K. and Patton, C.M. 1985 *Campylobacter*. In: Lennette E, Balows A, Hausler J, Shadon JrHJ, editors. Manual of clinical microbiology. Washington, D.C.: American Society for Microbiology. p302-308.

Moser, I., Schroeder, W. and Salnikow, J. 1997 *Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. FEMS Microbiol. Lett. 157: 233-238.

Nachamkin, I. and Barbagallo, S. 1990 Culture confirmation of *Campylobacter* spp. by latex agglutination. J. Clin. Microbiol. 28: 817-818.

Nachamkin, I., Engberg, J. and Aarestrup, F.M. 2000 Diagnosis and antimicrobial susceptibility of *Campylobacter* species. *In:* Campylobacter 2nd edition. Ed. I. Nachamkin & M.J. Blaser. ASM Press, Washington, USA, p45-66.

Nachamkin, I. 2002 Chronic effects of *Campylobacter* infection. Microbes Infect. 4: 399-403.

Nachamkin, I. 2003 *Campylobacter* and *Arcobacter*. In Manual of Clinical Microbiology. In: Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A., Yolken, R.H. (Eds.), American Society for Microbiology, Washington, DC, p902-914.

NACMCF, National Advisory Committee on Microbiological Criteria for Foods. 2007 The analytical utility of *Campylobacter* methodologies. J. Food Prot. 70: 241-250.

Nakari, U.M., Puhakka, A. and Siitonen, A. 2008 Correct identification and discrimination between *Campylobacter jejuni* and *C. coli* by a standardized hippurate test and species-specific polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. 27: 513-518.

Nannapaneni, R., Story, R., Wiggins, K.C. and Johnson, M.G. 2005 Concurrent quantitation of total *Campylobacter* and total ciprofloxacin-resistant *Campylobacter* loads in rinses from retail raw chicken carcasses from 2001 to 2003 by direct plating at 42 degrees C. Appl. Environ. Microbiol. 71: 4510-4515.

NARMS USFDA, National Antimicrobial Resistance Monitoring System/U.S. Food and Drug Administration. 2007Available at http://www.fda.gov/cvm/narms_pg.html. Accessed 2007Oct 20.

Nayak, R., Stewart, T.M. and Nawaz, M.S. 2005 PCR identification of *Campylobacter coli* and *Campylobacter jejuni* by partial sequencing of virulence genes. Mol. Cell. Probes. 19: 187-193.

Newell, D.G., McBride, H., Saunders, F., Dehele, Y. and Pearson, A.D. 1985 The virulence of clinical and environmental isolates of *Campylobacter jejuni*. J. Hyg.. 94: 45-54.

Newell, D.G. 2001 Animal models of *Campylobacter jejuni* colonisation and disease and the lessons to be learned from similar *Helicobacter pylori* models. J. Appl. Microbiol. 90: 57-67.

Ng, L.K., Sherburne, R., Taylor, D.E. and Stiles, M.E. 1985 Morphological forms and viability of *Campylobacter* species studied by electron microscopy. J. Bacteriol. 164: 338-343.

Nicholson, M.A. and Patton, C.M. 1995 Evaluation of disk method for hippurate hydrolysis by *Campylobacter* species. J. Clin. Microbiol. 33: 1341-1343.

Nogva, H.K., Bergh, A., Holck, A. and Rudi, K. 2000 Application of the 50-nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. Appl. Environ. Microbiol. 66: 4029-4036.

Nuijten, P.J., van Asten, F.J., Gaastra, W. and van der Zeijst, B.A. 1990 Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. 265: 17798-17804.

Oliphant, C.O. and Green, G.M. 2002. Quinolones: a comprehensive review. Clin. Pharmacol. 65: 455-464.

Oliver, J.D. 2005 The viable but nonculturable state in bacteria. J. Microbiol. 43: 93-100.

On, S.L. and Jordan, P.J. 2003 Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 41: 330-336.

Osano, O. and Arimi, S.M. 1999 Retail poultry and beef as sources of *Campylobacter jejuni*. East. Afr. Med. J. 76: 141-143.

O'Sullivan, N.A., Fallon, R., Carroll, C., Smith, T. and Maher, M. 2000 Detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in broiler chicken samples with a PCR/DNA probe membrane based colorimetric detection assay. Mol. Cell. Probes. 14, 7–16.

Oxoid Manual, 1998 The Oxoid Manual 8th ed. Bridson, E.Y. Oxoid Ltd. Basingstoke, England.

Oyofo, B.A., Thornton, S.A., Burr, D.H., Trust, T.J., Pavlovskis, O.R. and Guerry, P. 1992 Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by using polymerase chain reaction. J. Clin. Microbiol. 30: 2613-2619.

Oyofo, B.A., Abd el Salam, S.M., Churilla, A.M. and Wasfy, M.O. 1997 Rapid and sensitive detection of *Campylobacter* spp. from chicken using the polymerase chain reaction. Zentralbl. Bakteriol. 285: 480-485.

Oyarzabal, O.A., Backert, S., Nagaraj, M., Miller, R.S., Hussain, S.K. and Oyarzabal, E.A. 2007 Efficacy of supplemented buffered peptone water for the isolation of Campylobacter jejuni and C. coli from broiler retail products. J. Microbiol. Methods. 69: 129-136.

OzFoodNet network. Annual report 2007. 2006 Burden and causes of foodborne disease in Australia: Commun. Dis. Intell. 30: 278-300.

Padungton, P. and Kaneene, J.B. 2003 *Campylobacter* spp in human, chickens, pigs and their antimicrobial resistance. J. Vet. Med. Sci. 65: 161-170.

Padungtod, P. and Kaneene, J.B. 2005 *Campylobacter* in food animals and humans in northern Thailand. J. Food Prot. 68: 2519-2526.

Park, S.F. 2002 The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. Int. J. Food Microbiol. 74: 177-188.

Park, C.E. and Sanders, G.W. 1991 A sensitive enrichment procedure for the isolation of *Campylobacter jejuni* from frozen foods. In: G.M. Riuz-Palacios, F. Calva and B.R. Ruiz-Palacios, Editors, *Campylobacter V, Proc. 5th International Workshop on Campylobacter Infections*, National Institute of Nutrition, Puerto Vallarta, Mexico, p102.

Pearson, J.P., Kaura, R., Taylor, W. and Allen, A. 1982 The composition and polymeric structure of mucus glycoprotein from human gall-bladder bile. Biochim. Biophys. Acta. 706: 221-228.

Pearson, A.D., Greenwood, M.H., Donaldson, J., Healing, T.D., Jones, D.M., Shahamat, M., Feltham, R.K. and Colwell, R.R. 2000 Continuous source outbreak of campylobacteriosis traced to chicken. J. Food Prot. 63: 309-314.

Pebody, R.G., Ryan, M.J. and Wall, P.G. 1997 Outbreaks of *Campylobacter* infection: rare events for a common pathogen. Commun. Dis. Rep. CDR. Rev. 7: 33-37.

Pei, Z. and Blaser, M.J. 1993 PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. 268: 18717-18725.

Penner, J.L. and Hennessy, J.N. 1980 Passive hemagglutination technique for serotyping *Campylobacter fetus subsp. jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. 12: 732-737.

Phetsouvanh, R., Midorikawa, Y. and Nakamura, S. 1999 The seasonal variation in the microbial agents implicated in the etiology of diarrheal diseases among children

in Lao People's Democratic Republic. Southeast Asian J. Trop. Med. Public Health. 30: 319-323.

Pickett, C.L., Pescl, E.C., Cottle, D.L., Russell, G., Erdem, A.N. and Zeytin, H. 1996 Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. Infect. Immun. 64: 2070-2078.

Piddock, L.J.V., Ricci, V., Pumbwe, L., Everett, M.J. and Griggs, D.J. 2003 Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. J. Antimicrob. Chemother. 51: 19-26.

Post, D.E. 1995 Food-borne pathogens. Monograph Number 3: *Campylobacter*. Unipath Ltd, Basingstoke, UK, p1-33.

Purdy, D., Buswell, C.M., Hodgson, A.E., McAlpine, K., Henderson, I. and Leach, S.A. 2000 Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. J. Med. Microbiol. 49: 473-479.

Raben, D. and Slavik, M. 1994 Evaluation of oxyrase method for *Campylobacter* isolation from chicken carcasses. J. Rapid Methods Automation Microbiol. 2: 279-286.

Randall, L.P., Ridley, A.M., Cooles, S.W., Sharma, M., Sayers, A.R. and Pumbwe, L. 2003 Prevalence of multiple antibiotic resistance in 443 *Campylobacter* spp. isolated from humans and animals. J. Antimicrob. Chemother. 52: 507-510.

Rollins, D.M. and Colwell, R.R. 1986 Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52: 531-538.

Saha, S.K., Saha, S. and Sanyal, S.C. 1991 Recovery of injured *Campylobacter jejuni* cells after animal passage. Appl. Environ. Microbiol. 57: 3388-3389.

Said, B., Wright, F., Nichols, G.L., Reacher, M. and Rutter, M. 2003 Outbreaks of infectious disease associated with private drinking water supplies in England and Wales 1970–2000. Epidemiol. Infect. 130: 469-479.

Sails, A.D., Bolton, F.J., Fox, A.J., Wareing, D.R. and Greenway, D.L. 2002 Detection of *Campylobacter jejuni* and *Campylobacter coli* in environmental waters by PCR enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. 68: 1319-1324.

Sails, A.D., Fox, A.J., Bolton, F.J., Wareing, D.R. and Greenway, D.L. 2003 A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. Appl. Environ. Microbiol. 69: 1383-1390.

Sainsus, N., Cattori, V., Lepadatu, C. and Hofmann-Lehmann, R. 2008 Liquid culture medium for the rapid cultivation of *Helicobacter pylori* from biopsy specimens. Eur. J. Clin. Microbiol. Infect Dis. 27: 1209-1217.

Sallam, K.I. 2007 Prevalence of *Campylobacter* in chicken and chicken by-products retailed in Sapporo area, Hokkaido, Japan, Food Control. 18: 1113-1120.

Sambrook, J. and Russell, D.W. 2001 *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scawen, M. and Allen, A. 1977 The action of proteolytic enzymes on the glycoprotein from pig gastric mucus. Biochem. J. 163: 363-368.

Shih, D.Y. 2000 Isolation and identification of enteropathogenic *Campylobacter* spp. from chicken samples in Taipei. J. Food Prot. 63: 304-308.

Simpkins, S.A., Chan, A.B., Hays, J., Popping, B. and Cook, N. 2000 An RNA transcription-based amplification technique (NASBA) for the detection of viable Salmonella enterica. Lett. Appl. Microbiol. 30: 75-79.

Skirrow, M.B. 1977 *Campylobacter enteritis*: a new disease. Br Med J. 2: 9-11.

Smith, A., Reacher, M., Smerdon, W., Adak, G.K. and Nichols, G. 2006 Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. Epidemiol. Infect. 134: 1141-1149.

Snelling, W.J., Matsuda, M., Moore, J.E. and Dooley, J.S.G. 2005 Under the microscope *Campylobacter jejuni*. Lett. Appl. Microbiol. 41: 297-302.

Soderstrom, C., Schalen, C. and Walder, M. 1991 Septicaemia caused by unusual *Campylobacter* species (*C. laridis* and *C. mucosalis*). Scand. J. Infect. Dis. 23: 369-371.

Steele, T.W. and Mcdermott, S.N. 1984 The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. Pathology. 16: 263-265.

Steel, R.G.D. and Torrie, J.H. 1980 Principles and Procedures of Statistics, New York: McGraw-Hill.

Steinhauserova, I., Ceskova, J., Fojtikova, K. and Obrovska, I. 2001 Identification of thermophilic *Campylobacter* spp. by phenotypic and molecular methods. J. Appl. Microbiol. 90: 470-475.

Stern, N.J., Wojton, B. and Kwiatek, K. 1992 A differential-selective medium and dry icegenerated atmosphere for the recovery of *Campylobacter jejuni*. J. Food Prot. 55: 514-571.

Stern, N.J. 1994 Mucosal competitive exclusion to diminish colonisation of chickens by *Campylobacter jejuni*. Poult. Sci. 73: 402-407.

Sung, K., Hiett, K.L. and Stern, N.J. 2005 Heat-treated *Campylobacter* spp. and mRNA stability as determined by reverse transcriptase-polymerase chain reaction. Foodborne Pathog. Dis. 2: 130-137.

Suzuki, H. and Yamamoto, S. 2009 *Campylobacter* contamination in retail poultry meats and by-products in the world: a literature survey. J. Vet. Med. Sci. 71: 255-261.

Taboada, E.N., Luebbert, C.C. and Nash, J.H.E. 2007 Studying bacterial genome dynamics using microarray-based comparative genomic hybridization. In: Methods in Molecular Biology. Eds: Bergman NH. Human Press Inc., Totowa.

Talukder, K.A., Aslam, M., Islam, Z., Azmi, I.J., Dutta, D.K., Hossain, S., Nur-E-kamal, A., Nair, G.B., Cravioto, A., Sack, D.A. and Endtz, H.P. 2008 Prevalence of virulence genes and Cytolethal Distending Toxin (CDT) production in *Campylobacter jejuni* isolated from diarrheal patients in Bangladesh. J. Clin. Microbiol 4: 1485-1488.

Taylor, D.N., Perlman, D.M., Echeverria, P.D., Lexomboon, U. and Blaser, M.J. 1993 *Campylobacter* immunity and quantitative excretion rates in Thai children. J. Infect. Dis. 168: 754-758.

The Ministry of Health, Labour and Welfare. 2007 Statistics of Food Poisoning. http://www.mhlw.go.jp/topics/syokuchu/index.html.

The European Parliament and the Council of the European Union. Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. OJ L 325. 2003 Dec 12. p31-40.

Thomas, C., Hill, D. and Mabey, M. 2002 Culturability, injury and morphological dynamics of thermophilic *Campylobacter* spp. within a laboratory-based aquatic model system. J. Appl. Microbiol. 92: 433-442.

Tolcin, R., LaSalvia, M.M., Kirkley, B.A., Vetter, E.A., Cockerill, FR3rd. and Procop, G.W. 2000 Evaluation of the Alexon-trend ProSpecT *Campylobacter* microplate assay. J. Clin. Microbiol. 38: 3853-3855.

Totten, P.A., Patton, C.M., Tenover, F.C., Barrett, T.J., Stamm, W.E., Steigerwalt, A.G., Lin, J.Y., Holme, K.K. and Brennder, D.J. 1987 Prevalence and characterization of hippurate-negative *Campylobacter jejuni* in King County, Washington. J. Clin. Microbiol. 25: 1747-1752.

Tran, T.T. 1998 A blood-free enrichment medium for growing *Campylobacter* spp. under aerobic conditions. Lett. Appl. Microbiol. 26: 145-148.

Tran, J.H. and Jacoby, G.A. 2002 Mechanism of plasmid mediated quinolone resistance. PNAS. 99: 5638-5642.

Tu, Q.V., McGuckin, M.A. and Mendz, G.L. 2008 Campylobacter jejuni response to human mucin MUC2: modulation of colonization and pathogenicity determinants. J. Med. Microbiol. 57: 795-802.

US FDA, United States Food and Drug Administration., 2001 Bacteriological Analytical Manual, Chapter 7. *Campylobacter* (accessed 24 March 2008). Available from: http://www.cfsan.fda.gov/~ebam/bam-7.html.

Uyttendaele, M., Schukkink, R., van Gemen, B. and Debevere, J. 1995 Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). Appl. Environ. Microbiol. 61: 1341-1347.

Westrell, T., Ciampa, N., Boelaert, F., Helwigh, B., Korsgaard, H., Chríel, M., Ammon , A., Mäkelä, P. 2009 Zoonotic infections in Europe in 2007: a summary of the EFSA – ECDC anual report.

Vandamme, P. 2000 Taxonomy of the family Campylobacteriaceae. *In*: Campylobacter 2nd edition. Ed. I. Nachamkin and M.J. Blaser. ASM Press, Washington, USA, p3-26.

Vandenberg, O., Houf, K., Douat, N., Vlaes, L., Retore, P., Butzler, J.P. and Dediste, A. 2006 Antimicrobial susceptibility of clinical isolates of non-jejuni/coli

Campylobacter and arcobacters from Belgium. J. Antimicrob. Chemother. 57: 908-913.

Vandervliet, G.M., Schukkink, R.A., van Gemen, B., Schepers, P. and Klatser, P.R. 1993 Nucleic acid sequence-based amplification (NASBA) for the identification of mycobacteria. J. Gen. Microbiol. 139: 2423-2429.

van Doorn, L.J., Giesendorf, B.A., Bax, R., van der Zeijst, B.A., Vandamme, P. and Quint, W.G. 1997 Molecular discrimination between *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* by polymerase chain reaction based on a novel putative GTPase gene. Mol. Cell. Probes. 11: 177-185.

van Nierop, W., Dusé, A.G., Marais, E., Aithma, N., Thothobolo, N., Kassel, M., Stewart, R., Potgieter, A., Fernandes, B., Galpin, J.S. and Bloomfield, S.F. 2004 Contamination of chicken carcasses in Gauteng, South Africa, by *Salmonella*, *Listeria monocytogenes* and *Campylobacter*. Int. J. Food Microbiol. 99: 1-6.

van Vliet, A.H.M. and Ketley, J.M. 2001 Pathogenesis of enteric *Campylobacter* infection. J. Appl. Microbiol. 90: 45-56.

Varavithya, W., Vathanophas, K. and Bodhidatta, L. 1990 Importance of *Salmonellae* and *Campylobacter jejuni* in the etiology of diarrheal disease among children less than 5 years of age in a community in Bangkok, Thailand. J. Clin. Microbiol. 28: 2507-2510.

Vindigni, S.M., Srijan, A., Wongstitwilairoong, B., Marcus, R., Meek, J., Riley, P.L. and Mason, C. 2007 Prevalence of foodborne microorganisms in retail foods in Thailand. Foodborne Pathog. Dis. 4: 208-215.

Waage, A.S., Varund, T., Lund, V. and Kapperud, G. 1999 Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. Appl. Environ. Microbiol. 65: 1636-1643.

Wang, G., Clark, C.G., Taylor, T.M., Pucknell, C., Barton, C., Price, L., Woodward, D.L. and Rodgers, F.G. 2002 Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus subsp. fetus*. J. Clin. Microbiol. 40: 4744-4747.

Waino, M., Bang, D.D., Lund, M., Nordentoft, S., Aadersen, J.S., Pedersen, K. and Madsen, M. 2003 Identification of campylobacteria isolated from Danish broilers by phenotypic tests and species-specific PCR assays. J. Appl. Microbiol. 95: 649-655.

Wallace, R.B. 1997 *Campylobacter*. In: Foodborne microorganisms of public health significance, 5th Edition. Eds., Hocking, A.D., Arnold, G., Jenson, I., Newton, K. & Sutherland P. AIFST (NSW Branch) Food Microbiology Group, CSIRO Food Science & Technology, Trenear Printing Service Pty. Ltd, Tempe, NSW, Australia, p267-284.

Wardak, S. and Szych, J. 2006 Prevalence of pathogenic genes of *Campylobacter jejuni* isolated from humans in Poland between 2003-2005. Med. Dosw. Mikrobiol. 58: 217-222.

Wassenaar, T.M., Bleumink-Pluym, N.M.C. and van der Zeijst, B.A.M. 1991 Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. EMBO J. 8: 2055-2061.

Wassenaar, T.M. and Blaser, M.J. 1999 Oct Pathophysiology of *Campylobacter jejuni* infections of humans. Microbes Infect. 1: 1023-1033.

Werno, A., Klena, J.D., Shaw, G.M. and Murdoch, D.R. 2002 Fatal case of *Campylobacter lari* prosthetic joint infection and bacteremia in an immunocompetent patient. J. Clin. Microbiol. 40: 1053-1055.

Whitehouse, C.A., Balbo, P.B., Pesci, E.C., Cottle, D.L., Mirabito, P.M. and Pickett. C.L. 1998 *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect. immin. 66: 1934-1940.

WHO, World Health Organization. 2007 Fact Sheet No. 237: Food safety and foodborne illness. Available from: http://www.who.int/mediacentre/factsheets/fs237/en. Accessed 2008Sep.

Williams, L.K., McMeechan, A., Baalham, T., Ward, L., Humphrey, T.J. and Jørgensen, F. 2008 Detection of *Campylobacter jejuni* and *Campylobacter coli* from broiler chicken-related samples using BAX PCR and conventional International Organization for Standardization culture. J. Food Prot. 71: 835-838.

Wonglumsom, W., Vishnubhatla, A., Kim, J.M. and Fung, D.Y. 2001 Enrichment media for isolation of *Campylobacter jejuni* from inoculated ground beef and chicken skin under normal atmosphere. J. Food Prot. 64: 630-634.

Woolridge, K.G. and Ketley, J.M. 1997 *Campylobacter*-host cell interactions. Trends Microbiol. 5: 96-102.

Wösten, M.M.S.M., Wagenaar, J.A. and van Putten J.P.M. 2004 The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. J. Biol. Chem. 279: 16214-16222.

Xing, J.M., Zhang, S., Zhang, H.H., Shen, C.F., Bi, D., Li, G. and Yao, L.H. 2008 Detection and identification of seven clinical common pathogenic bacteria by oligonucleotide microarray. Zhonghua Liu Xing Bing Xue Za Zhi. 29: 15-18.

Yang, C., Jiang, Y., Huang, K., Zhu, C. and Yin, Y. 2003 Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. FEMS Immunol. Med. Microbiol. 38: 265-271.

Yao, R., Burr, D.H., Doig, P., Trust, T.J., Niu, H. and Guerry, P. 1994 Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. Mol. Microbiol. 14: 883-893.

Yao, R., Burr, D.H. and Guerry, P. 1997 CheY-mediated modulation of *Campylobacter jejuni* virulence. Mol. Microbiol. 23: 1021-1031.

You, Y., Fu, C., Zeng, X., Fang, D., Yan, X., Sun, B., Xiao, D. and Zhang, J. 2008 A novel DNA microarray for rapid diagnosis of enteropathogenic bacteria in stool specimens of patients with diarrhea. J. Microbiol. Methods. 75: 566-571.

Zhang, H., Gong, Z., Pui, O., Liu, Y. and Li, X.F. 2006 An electronic DNA microarray technique for detection and differentiation of viable *Campylobacter* species. Analyst. 131: 907-915.

Zhang, L., Man, S.M., Day, A.S., Leach, S.T., Lemberg, D.A., Dutt, S., Stormon, M., Otley, A., O'Loughlin, E.V., Magoffin, A., Ng, P.H.Y. and Mitchell, H. 2009 Detection and isolation of *Campylobacter* species other than *C. jejuni* from children with Crohn's disease. J. Clin. Microbiol. 47: 453-454.

Zhang, M.J., Gu, Y.X., Ran, L. and Zhang, J.Z. 2007 Multi-PCR identification and virulence genes detection of *Campylobacter jejuni* isolated from China. Zhonghua Liu Xing Bing Xue Za Zhi. 28: 377-380.

Zirnstein, G., Li, Y., Swaminathan, B. and Angulo, F. 1999 Ciprofloxacin resistance in *Campylobacter jejuni* isolates: Detection of gyrA resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. J. Clin. Microbiol. 37: 3276-3280.

VITAE

Name Mr. Sueptrakool Wisessombat

Student ID 4823024

Educational Attainment

Degree	Name of Institution	Year of Graduation
B. Sc. (Medical technology)	Walailak University	2002
M. Sc. (Microbiology)	Prince of Songkla University	2005

Work-Position and Address

Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand

List of Publications and Proceedings

Publications

1. **Wisessombat**, **S**., Kittiniyom, K., Srimanote, P., Wonglumsom, W. and Voravuthikunchai, S.P. 2009 Enhancement of viable *Campylobacter* detection by chemotactic stimuli. Lett. Appl. Microbiol. (Submitted LAM-2009-1418).
2. **Wisessombat**, **S**., Kittiniyom, K., Srimanote, P., Wonglumsom, W. and Voravuthikunchai, S.P. 2009 Multiplex PCR for direct identification of thermophilic *Campylobacter***,** *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* and simultaneous detection of *cdtB* gene. J. Rapid Methods Autom. Microbiol. (In press).
3. **Wisessombat**, **S**., Kittiniyom, K., Srimanote, P., Wonglumsom, W. and Voravuthikunchai, S.P. 2009 A novel method and simple apparatus for the detection of thermophilic *Campylobacter* spp. in chicken meat products. J. Microbiol. Methods. 76: 169-173.

Proceedings

1. Voravuthikunchai, S.P. and **Wisessombat**, **S**. 2009 Enhancement of motility ability of viable thermophilic *Campylobacter* in viscous condition. Programme and Abstracts, The 19th European Congress of Clinical Microbiology and Infectious Diseases, 16-19 May, 2009 at Helsinki**,** Finland.

2. Sirirak, T., **Wisessombat**, **S**. and Voravuthikunchai, S.P. 2008 Antibacterial activity of Thai medicinal plants against *Campylobacter* spp. isolated from chickens. International Conference on Natural Products for Health and Beauty, 17-19 Dec, 2008 at Phayao, Thailand.

Patents

1. **Wisessombat**, **S**., Kittiniyom, K., Srimanote, P., Wonglumsom, W. and Voravuthikunchai, S.P. 2008 A rapid detection method for viable *Campylobacter*. Thai Patent 0801000381, January 25.