3
2002

This is to certify that the

dissertation entitled

EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF "CAMPYLOBACTER SPP." FROM FOOD ANIMALS AND HUMANS IN NORTHERN THAILAND

presented by

PAWIN PADUNGTOD

has been accepted towards fulfillment of the requirements for

DOCTORAL degree in LARGE ANIMAL CLINICAL SCIENCES (EPIDEMIOLOGY)

JB Kaneene

Major professor

PROF JOHN B KANEENE

Date 5/7/02

EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER SPP.* IN FOOD ANIMALS AND HUMANS IN NORTHERN THAILAND

By

Pawin Padungtod

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Large Animal Clinical Sciences
(Epidemiology)

2002

ABSTRACT

EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER SPP.* IN FOOD ANIMALS AND HUMANS IN NORTHERN THAILAND

BY

Pawin Padungtod

Campylobacter spp. have been recognized as major foodborne pathogens in developed and developing countries. Recently, *Campylobacter spp.* with resistance to antimicrobial agents have been identified in various parts of the world, including Thailand. It is also widely speculated that the use of antimicrobial agents in food animals may be contributing to the antimicrobial resistance problem in humans. Because there was limited information on *Campylobacter spp.* in food animals in Thailand, and to determine whether food animals were an important source of *Campylobacter spp.* with resistance to antimicrobial agents, a study was designed to address six objectives: 1) validate the use of a fluorogenic PCR assay to identify *C. jejuni* from field samples; 2) determine the frequencies and the antimicrobial susceptibility level of *Campylobacter spp.* isolated from food animals and farm workers at farms, slaughterhouses, and markets; 3) compare the frequencies of *Campylobacter spp.* with resistance to antimicrobial agents in food animals, food products and farm workers; 4) determine what risk factors associated with the observed frequencies of antimicrobial resistance; 5) determine whether antimicrobial use in feed and treatment on pig and chicken farms is associated with the frequency of antimicrobial resistance in *Campylobacter*; and 6) determine the association between mutation in the *gyrA* gene of *C. jejuni* and level of resistance to ciprofloxacin.

A combination of cross-sectional and prospective study designs were used. Samples were collected from pigs and chickens at the farm, slaughterhouse, and market in 2000 and 2001. Farm and slaughterhouse worker stool samples were also collected. Isolation of *Campylobacter spp.* was done using enrichment and selective media, and suspect colonies were confirmed using oxidase test, catalase test and gram staining. In vitro susceptibility testing was done using the microbroth dilution technique, for ciprofloxacin, erythromycin, gentamycin, azithromycin, clindamycin, chloramphenicol, nalidixic acid, and tetracycline. Results of susceptibility testing were reported in minimum inhibitory concentrations (MICs), and isolates were classified as resistant based on breakpoints from the National Committee for Clinical Laboratory Standards. Fluorogenic PCR was used to identify *C. jejuni* and the Thr-86-to-Ile mutation in the *gyrA* gene of *C. jejuni*.

The prevalence of *Campylobacter spp.* in pigs was found to be 73.6% at the farm, 45.6% at the slaughterhouse, and 24.6% at the market. In chickens, the prevalence was 73.4% at the farm, 50.6% at the slaughterhouse, and 47.2% at the market. In humans, the prevalences of *Campylobacter spp.* were12.5% and 0% in pig farm workers and chicken farm workers, respectively. Resistance was found to all antimicrobial agents tested in chickens and pigs at farms, slaughterhouses and markets. The most prevalent forms of resistance seen were to ciprofloxacin, nalidixic acid, and tetracycline. The prevalence of resistance was higher for those antimicrobial agents to which animals were exposed. There was very high agreement between ciprofloxacin resistance and the presence of the Thr-86-to-Ile mutation in *gyrA* gene.

ACKNOWLEDGMENTS

I would like to thank the following institutions for their generosity; Institute of International Health, MSU, National Food Safety and Toxicology Center, MSU, Population Medicine Center, MSU, and Chiang Mai University, Chiang Mai, Thailand for financial support, Faculty of Veterinary Medicine, Chiang Mai University for technical assistance.

I would like to thank all my committee members, graduate students, technicians and secretaries in all laboratories I worked in including AHDL bacteriology lab, Dr.Holland's E.coli lab, Dr.Linz's lab, Dr.Mansfield's lab, Faculty of Medicine-Chiang Mai University bacteriology lab, and Faculty of veterinary medicine-Chiang Mai university central diagnostic lab, and all the students who helped me with specimen collection and laboratory work.

Special thanks to Prof. John Kaneene who has been a great mentor, and one of the persons I like working with most. He constantly shows me the power of optimism and the power of seeing thing in epidemiological way. Special thanks also to graduate students and secretaries in the Population Medicine Center. If carrying out a research study is like going to war, Prof. Kaneene would be a great general, and the Population Medicine Center would be a great company that I feel most comfortable going to war with.

Finally, I would like to thank the 3 most important women in my life. My mother, who built in me the love to read and to believe in education. My sister, who is always my gold standard of doing things. And my wife, who makes it easy for me to be happy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

RATIONALE

Foodborne diseases have recently emerged as major public health issues around the world. The consequences of infection by foodborne bacteria may vary depending on the specific agent and characteristic of the patient. The most important consequence of foodborne bacteria affecting millions of people each year is gastroenteritis or diarrhea. Diarrhea is one of the top causes of mortality in children in developing world, particularly in children less than one year old.

Until recently, *Salmonella* and *Shigella* were the most important foodborne pathogens, in terms of virulence and the number of people affected. Laboratory methods and surveillance systems for those two pathogens were established in developed and developing countries. However, since the early 1990s, *Campylobacter spp.*, especially *C. jejuni* and *C. coli,* have emerged as major foodborne pathogens, with higher incidences than *Salmonella* and *Shigella,* and foods of animal origin have been implicated as a major source of *Campylobacter spp.* infection in humans. Recently, there have been major improvements in detection and identification techniques for *Campylobacter spp.*, and surveillance systems for *Campylobacter spp.* have been put in place in many developed nations. However, there is very limited information regarding the epidemiology of *Campylobacter spp.* in developing nations.

The scope of food borne bacteria nowadays can be best viewed as an international issue, considering the number of travelers and trades of food commodities around the world. Bacteria originating in one part of the world may easily end up causing disease in place far away from its' origin. Clarifying the epidemiology of *Campylobacter spp.* in

developing countries will contribute to the global picture of food borne disease resulting from *Campylobacter spp.* infection. This clarification will benefit both producers and consumers of foods of animal origin around the world.

Another major public health issue at the turn of the century is the emergence of food borne bacteria with resistance to antimicrobial agents. The fact that bacteria can develop resistance to antimicrobial agents was recognized a long time ago. However, most of those cases were limited to bacterial isolates found in hospitals or health settings, which were constantly exposed to antimicrobial agents. The impact of those nosocomial infections with resistance bacteria was limited to those requiring hospitalization. Now that food borne bacteria have also been found with resistance to antimicrobial agents, the impact may be far greater than those nosocomial pathogens.

It is widely speculated that antimicrobial used in food animal production contribute significantly to the development of food borne bacteria with resistance to antimicrobial agents. One of the reasons was that amount of antimicrobial agents used as growth promoters in food animal production far exceed those used in human medicine. In developed countries, antimicrobial usage in humans and animals are strictly regulated, whereas in developing countries, antimicrobial usage is not strictly regulated. Therefore, the prevalence of resistance bacteria can be expected to be higher in developing countries. With the loose control of antimicrobial usage in human and animals and the large size of its food animal industry, Thailand offers a unique opportunity to study this problem.

There is a critical need for epidemiological information on foodborne bacteria with resistance to antimicrobial agents from developing countries. Apart from public health concerns, the increasing trade in foods of animal origin around the world makes the safety of such foods an important issue for food-producing countries such as Thailand. The information will be important for regulatory agencies overseeing the use of antimicrobial agents in food animals and humans, and agencies overseeing the trade of foods from animal origin.

PROBLEM STATEMENT

Despite the public health and economic significance of *Campylobacter spp.* in food animals in Thailand, there is only a limited base information available. *Campylobacter spp.* with resistance to antimicrobial agents have been reported in both developed and developing countries, and the prevalence of potentially antimicrobial-resistant *Campylobacter spp.* in food animals indicates that foods of animal origin may be a source of resistant bacteria to humans. Consequently, there is an urgent need to fill this gap in knowledge.

OBJECTIVES

In order to determine whether food animals are an important source of antimicrobial-resistant bacteria to humans, a thorough, stepwise research approach is needed. This thesis focuses on the epidemiology and antimicrobial resistance of *Campylobacter spp.* in food animals and humans in Thailand. The main objectives of the study were to:

1. Validate the use of a fluorogenic PCR assay to identify *C. jejuni* from field samples.
2. Determine the frequencies and the antimicrobial susceptibility level of *Campylobacter spp.* isolated from food animals and workers at pig and chicken farms, slaughterhouses, and markets.
3. Compare the frequencies of *Campylobacter spp.* with resistance to antimicrobial agents in food animals, food products and farm workers.
4. Determine the risk factors associated with the observed frequencies of *Campylobacter spp.* and frequencies of *Campylobacter spp.* with resistance to antimicrobial agents isolated from various sources.
5. Determine whether antimicrobial use in feed and treatment on pig and chicken farms is associated with the frequency of antimicrobial resistance in *Campylobacter.*
6. Determine the association between mutation in *gyrA* gene of *Campylobacter jejuni* and ciprofloxacin resistance.

HYPOTHESES

The following hypotheses were tested:

1. A fluorogenic PCR assay can be used to identify *C. jejuni* from field samples, providing results comparable to conventional phenotypic tests.
2. *Campylobacter spp.* are prevalent in pig and chicken production systems in northern Thailand.
3. Major factors influencing the observed prevalence of *Campylobacter spp.* in pig and chicken production systems can be determined.

4. *Campylobacter spp.* with resistance to antimicrobial agents are prevalent throughout the pig and chicken production systems and farm and slaughterhouse workers in northern Thailand.
5. Antimicrobial use in pig and chicken production systems is associated with the frequency of antimicrobial resistance in *Campylobacter* isolated from these animals and farm workers.
6. The Thr-86-to-Ile mutation in the *gyrA* gene of *C. jejuni* is associated with resistance to ciprofloxacin.

OVERVIEW

Chapter 1 is a literature review of the epidemiology and antimicrobial resistance of *Campylobacter spp.* in humans and food animals, focusing on comparisons between developed and developing countries. Chapter 2 is a literature review of molecular techniques used in epidemiological studies of *Campylobacter spp.*, focusing on the available molecular techniques and application of those techniques.

Chapter 3 addresses Hypothesis 1, by reporting a laboratory test of a fluorogenic PCR assay in comparison with conventional phenotypic tests. Chapters 4 and 5 address Hypotheses 2 and 3, and describe results of an epidemiological study of *Campylobacter spp.* in pig, chicken and farm workers. Chapter 6 addresses Hypotheses 4 and 5, which involve an epidemiological study of antimicrobial resistance in *Campylobacter spp.* from pigs, chickens, and farm workers. Chapter 7 addresses Hypothesis 6, reporting a laboratory study of the mechanism of fluoroquinolone resistance in *C. jejuni.* The last section of this thesis is a summary of the results of these results.

CHAPTER 1

CAMPYLOBACTER SPP., AND THEIR ANTIMICROBIAL RESISTANCE, IN HUMANS, CHICKENS, AND PIGS : A REVIEW

INTRODUCTION

Campylobacter spp. are gram-negative, non-spore forming curved or spiral bacilli which grow under microaerophilic conditions. The first *Campylobacter* may have been isolated in 1913, but were classified as *Vibrio* spp. until the genus *Campylobacter* was established in 1963. Currently, the Campylobacteriaceae include the genera *Campylobacter* and *Arcobacter* (Vandamme, 2000), with the related genera *Helicobacter* and *Wolinella* in the novel family Helicobacteriaceae (Vandamme, 2000). There are 14 species of *Campylobacter* (Vandamme, 2000), of these *C. jejuni* is frequently associated with human gastroenteritis (Friedman, et al., 2000), and *C. fetus* subsp. *fetus* and *venerealis* are important animal pathogens (Garcia, et al., 1983).

The purpose of this literature review was to compare *Campylobacter spp.* in food animals and humans in developed and developing countries. In particular, comparisons were made on: 1) prevalence/incidence of campylobacteriosis, 2) major means of exposure and transmission, 3) clinical disease expression, and 4) problems of antimicrobial resistance observed in these organisms, including factors associated with the occurrence and development of resistance. It is believed that a comparative analysis of the literatures will provide some insight into identifying gaps in our knowledge regarding the epidemiology of *Campylobacter* infections and associated disease, different ways to control the problem, and suggest critical areas for future research.

PREVALENCE AND INCIDENCE OF *CAMPYLOBACTER SPP.*

***Campylobacter* in Humans.** Of all *Campylobacter* spp., *C. jejuni* is the species most frequently isolated in cases of human infection (Tay, et al., 1995). In developing countries, most reported *Campylobacter* infections are in children. Peaks in *Campylobacter* infection rates have been reported in children less than one year of age (Friedman, et al., 2000), and children less than 5 years old in Southeast Asia (Oberhelman and Taylor, 2000). Previously reported prevalences of *Campylobacter spp.* in children in Southeast Asia range from 2.9% to 15% (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988; Taylor, et al., 1993; Varavithya, et al., 1990). In children in Thailand, *Campylobacter spp.* and *Salmonella* are common causes of diarrhea (Rasrinual, et al., 1988; Varavithya, et al., 1990), and co-infection with *Campylobacter* and *E. coli*, *Salmonella* or *Shigella* is common (Poocharoen and Bruin, 1986). This exposure to *Campylobacter spp.* early in life, and reports of levels of *Campylobacter*-specific antibodies increasing with age (Taylor, et al., 1993), may result in less severe clinical symptoms in adults, which makes detection and reporting of cases rare. Since *Campylobacter* is commonly isolated with other enteric pathogenic bacteria in developing countries, reports of *Campylobacter* cases may be largely underestimated. There have been no reports of seasonal patterns in the occurrence of human *Campylobacter* infection in developing countries

In developed countries, *Campylobacter spp.* have been identified as etiologic agents in outbreaks and sporadic cases of gastroenteritis (Lehner, et al., 2000). The reported incidence of *Campylobacter* infection in U.S. was 20.1 cases per100,000 in 2000 (Acheson, 2001), and from 60 to 90 cases per 100,000 in northern Europe (Friedman, et

al., 2000). When taking under-reporting into consideration, the true incidence rate is estimated to be 1,000 - 2,300 cases per 100,000 in Europe (Friedman, et al., 2000). *Campylobacter* affects all age groups in developed countries, with one peak in children less than 4 years old, and in young adults from age 15 to 44 (Friedman, et al., 2000). The incidence of *Campylobacter* in developed countries also showed seasonal patterns, with peaks during the warmer months of the year (Friedman, et al., 2000), which may be a result of increasing survival of *Campylobacter* in the environment in warm weather.

The frequency and pattern of occurrence of *Campylobacter spp.* differ between developed and developing countries, especially in the number of cases reported in adults and the presence of any seasonal patterns in occurrence. It should be noted that there is limited information on the incidence of *Campylobacter* infections from developing countries. Most developed countries in Europe and North America have surveillance programs for *Campylobacter* and other foodborne pathogens, such as FoodNet in the U.S. (Acheson, 2001). Establishment of similar surveillance programs would be beneficial in developing countries.

***Campylobacter* in Food Animals.** Many different animal species maintain *Campylobacter spp.* with no clinical signs (Steinhauserova, et al., 2001). The most important species of *Campylobacter* in veterinary medicine are *C. fetus* subsp. *fetus* and *venerealis* (Garcia, et al., 1983). Of *Campylobacter* spp. that are pathogenic in food animals, *C. fetus* can cause reproductive disorders in cattle and sheep (Quinn, et al., 1994), and *C. hyointestinalis* and *C. mucosalis* have been associated with enteritis in pigs and cattle (Garcia, et al., 1983).

There is limited information on *Campylobacter spp.* in food animals or foods of animal origin from developing countries. In Thailand, *Campylobacter spp.* was isolated from 12% of various food samples including pork, chicken and vegetables in Bangkok (Rasrinual, et al., 1988), 40% of poultry ceca in India (Das, et al., 1996), and 68-100% of poultry samples from retail markets in Taiwan (Shih, 2000). The low prevalence of *Campylobacter* reported in Thailand was probably due to the fact that various types of meats and vegetable were included in the study, while the other two studies examined only poultry products. *Campylobacter* was isolated more in samples from fresh markets than in supermarkets in Taipei, Taiwan (Shih, 2000). In Kenya, *Campylobacter* was found on 77% of poultry products at the market (Osano and Arimi, 1999), while the prevalence of *Campylobacter* at the poultry farm was found to be 64-100% (Kazwala, et al., 1990; Simango and Rukure, 1991). By species, *C. jejuni* was isolated more frequently than *C. coli* in live chickens in Africa (Simango and Rukure, 1991).

There have been many studies on *Campylobacter spp.* in food animals, and foods of animal origin, in developed countries. In the past, research in cattle was limited, but has been increasing as outbreaks of human campylobacteriosis have been traced to foods of cattle origin (Kalman, et al., 2000). The prevalence of *Campylobacter* was found to be 15% in beef calves (Busato, et al., 1998), 37.7% in dairy herds (Wesley, et al., 2000), and 89.4% on beef cattle at slaughter (Stanley, et al., 1998). The prevalence in beef cattle at slaughter peaked in the summer (Stanley, et al., 1998), which coincides with the seasonal peak of human *Campylobacter* infections (Friedman, et al., 2000).

Extensive research has been conducted on *Campylobacter* in pigs. Pigs carry

higher proportions of *C. coli* than *C. jejuni*, whether they have enteritis or not (Harvey, et al., 1999; Steinhauserova, et al., 2001). In the U.S., *Campylobacter spp.* was isolated from 76% of gilts, 100% of pregnant sows, 57.8% of newborn piglets, and 100% of weaning pigs (Young, et al., 2000). Fifty percent of piglets were infected with the same serotype as their sows by seven days of age in the Netherlands (Weijtens, et al., 1997). The average number of *Campylobacter* colonizing the gut decreased toward the end of the rearing period (Weijtens, et al., 1999). A study in Belgium reported the prevalence of *Campylobacter spp.* on pig carcasses at slaughterhouses to be 2% (Korsak, et al., 1998), and *Campylobacter* was found in 1.3% of samples from pork from a retail market in the U.S. (Duffy, et al., 2001).

There is a large body of research on *Campylobacter* in poultry. The reported prevalence of *Campylobacter* in broiler flocks ranged between 35-57% in Europe (Atanassova and Ring, 1999) (Pearson, et al., 1996; Refregier-Petton, et al., 2001; Van de Giessen, et al., 1996). The prevalence of *Campylobacter* colonization was higher in organic farms (100%) when compared to extensive indoor system (49.2%) or conventional farms (36.7%) (Heuer, et al., 2001). A study at Swedish slaughterhouses showed a 27% flock prevalence, which increased with bird age and flock size (Berndtson, et al., 1996). The prevalence of infection in chickens has been shown to increase linearly with the age of the birds (Genigeorgis, et al., 1986). At broiler farms, day-old chickens can be colonized with *Campylobacter* (Stern, et al., 2000), with rates of 100% colonization by 3-4 weeks (Jacob-Reitsma, et al., 1995). Up to 40% of the flocks in the U.K. were found to be colonized with *Campylobacter spp.* by four weeks, and up to 90% by seven weeks (Evans and Sayers, 2000). After transportation, counts of

Campylobacter in chickens increased (Stern, et al., 1995; Whyte, et al., 2001), although there was no significant increase in prevalence (Whyte, et al., 2001). The reported prevalence of *Campylobacter spp.* in retail chickens varied from 28.5-100% (Table 1-1). Lower levels of contamination were found in chicken pieces without skin (Berrang, et al., 2001; Uyttendaele, et al., 1999), but removal of skin did not change the prevalence of *Campylobacter spp.* found on chicken carcasses (Berrang, et al., 2001). Frozen chickens had lower bacteria counts than fresh chickens (Dufrenne, et al., 2001; Stern, et al., 1984). The highest levels of recovery usually occurred during the warmer months (Jun - Oct) (Jacob-Reitsma, et al., 1994; Stern, et al., 1985; Wedderkopp, et al., 2000; Willis and Murray, 1996), and more *C. jejuni* (43-86%) than *C. coli* (11-39%) were recovered (Atanassova and Ring, 1999; Jacob-Reitsma, et al., 1994; Shih, 2000; Wedderkopp, et al., 2000). The seasonality of *Campylobacter* found in poultry products at the market coincide with the peak of incidence of *Campylobacter* in humans, which demonstrates the importance of chicken as a source of *Campylobacter* infection for humans in developed countries.

There do not appear to be significantly different colonization rates of *Campylobacter* in food animals between developed and developing countries. However, since the housing and management of food animals differs between developing and developed countries, the rate of colonization in developing countries, which are mostly located near the equator, should be higher than those in developed countries. This speculation should be confirmed by field observation.

EXPOSURE AND TRANSMISSION OF *CAMPYLOBACTER*

In Humans. In developed countries, *Campylobacter spp.* are associated with sporadic cases and outbreaks of infection. Outbreaks of *Campylobacter* are usually associated with raw milk (Altekruse, et al., 1998; Kalman, et al., 2000), whereas sporadic illnesses are often associated with consumption of chickens (Deming, et al., 1987; Effler, et al., 2001; Kapperud, et al., 1992; Schorr, et al., 1994; Studahl and Andersson, 2000). Other reported risk factors for *Campylobacter spp.* infection included handling of chickens or preparing chickens (Hopkins and Scott, 1983), contact with cats (Deming, et al., 1987), consuming antibiotics before illness (Effler, et al., 2001), eating pork (Studahl and Andersson, 2000), barbequing (Kapperud, et al., 1992; Studahl and Andersson, 2000), living or working on farms (Studahl and Andersson, 2000), working in slaughterhouses (Cawthraw, et al., 2000), exposure to animals with diarrhea (Saeed, et al., 1993), and travel abroad (Schorr, et al., 1994). Traveling to developing countries was associated with *Campylobacter* with resistance to antimicrobial drugs (Gaunt and Piddock, 1996). Foreigners residing in countries where *Campylobacter* is prevalent also have high risks of infection (Gaudio, et al., 1996). In addition to traditional outbreak investigational techniques, various molecular identification techniques are now available for epidemiological typing of *Campylobacter spp.*(Wassenaar and Newell, 2000). These techniques have been used to trace outbreaks back to broiler or dairy farm and food handlers (Kalman, et al., 2000; Olsen, et al., 2001; Pearson, et al., 2000). A recent molecular study also suggested a link between the *Campylobacter spp.* found in farm environments and those causing diseases in local communities (Fitzgerald, et al., 2001).

In developing countries, chicken products were found to be an important source of *Campylobacter* in humans in both Asia (Rasrinual, et al., 1988) and Africa (Simango and Rukure, 1991), and chickens were also found to be a potential source of *Campylobacter* in farm workers (Simango and Rukure, 1991). Although higher prevalences of *Campylobacter* on poultry products was reported in developing countries, the local customs of eating well-cooked meat and poultry may reduce the risk of infection, when compared to developed countries, where the consumption of undercooked meat is more common.

In Food Animals. There have been several studies on the epidemiology of *Campylobacter* in poultry in developed countries. Sources of infection were more likely to be horizontal contamination from the environment (Jacob-Reitsma, 1997; Van de Giessen, et al., 1992) or water system (Kazwala, et al., 1990; Pearson, et al., 1993), rather than from direct flock-to-flock transmission (Pearson, et al., 1996). Although prevalence of *Campylobacter spp.* is high (67%) in breeder flocks, serotyping of the organism did not support the hypothesis of vertical transmission (Chuma, et al., 1997; Jacob-Reitsma, 1995; Petersen, et al., 2001). Additional risk factors for *Campylobacter* colonization include housing with no air circulation, more than two workers working in the same poultry house, more than three poultry houses, acidified drinking water, the presence of insects or litter in worker changing rooms (Refregier-Petton, et al., 2001), batch depletion of the flock (not all in/all out) (Hald, et al., 2001), and the presence of other animals (pig, cattle, sheep) on the farm (Van de Giessen, et al., 1996). Wild birds have been found with *Campylobacter spp.*, with prevalences up to 50% in birds near chicken houses

(Craven, et al., 2000) and 40% in dead wild birds found in broiler houses (Jones, et al., 1991), and has been found in wild birds not associated with poultry facilities (Chuma, et al., 2000).

A longitudinal study that followed the same flock of chickens from farm to processing plant and market found the highest prevalence of *Campylobacter* at the processing plant (32.5%), especially on carcasses after chilling (52%), suggesting that contamination of carcasses most likely occurred at the processing plant(Jones, et al., 1991). Counts of *Campylobacter* were highest in caeca and colon of birds at slaughter (Berrang and Dickens, 2000), and the number of *Campylobacter* declined after scalding, but increased during picking and evisceration (Berrang and Dickens, 2000; Whyte, et al., 2001), which suggested that the caecum and colon are the most probable sources of contamination in the slaughterhouses, which has been confirmed by molecular typing (Newell, et al., 2001). After processing contaminated flocks, *Campylobacter spp.* can be found on all slaughter equipment (Berndtson, et al., 1996; Newell, et al., 2001), making cross-contamination between different flocks at slaughter possible (Newell, et al., 2001; Rivoal, et al., 1999). Other risk factors for the isolation of *Campylobacter* at slaughterhouses include short empty period between flocks, the lack of strict barriers such as changing boots between houses, wet litter or bedding, the presence of other poultry on the farm, staff handling other poultry on the farm, dividing the flock before slaughter, slaughterhouse staff loading birds from several different farms, and the presence of mice (Berndtson, et al., 1996).

In developed countries, the permanent colonization of *Campylobacter spp.* in pigs is probably related to constant exposure to other colonized pigs, since experiments have shown that pigs reared off-sow had less prevalence of *Campylobacter spp.* (Harvey, et al., 2000).

Very little research has been done on the epidemiology of *Campylobacter* in food animals in developing countries. In one study, *Campylobacter* were isolated frequently from surface water and foot baths, which may be a potential source of *Campylobacter* on the farm (Kazwala, et al., 1990). Because of the differences in the rearing systems and the slaughtering processes, risk factors for *Campylobacter* contamination in food animals in developing countries may be significantly different from those in developed countries. Some factors on the farm, such as biosecurity measures and the presence of wild birds, may be similar between developed and developing countries. In many developing countries, the majority of the slaughterhouses that process meat for local consumption commonly have lower standards of hygiene, which may also result in higher prevalence of *Campylobacter* on their products. However, meat in local markets in developing countries usually does not remain in the market more than one day after slaughter, so bacteria do not have the opportunities to multiply as much as in developed countries, especially for slow-growing organism like *Campylobacter*. Epidemiological studies of *Campylobacter* in food animals under different rearing systems in developing countries should be conducted to clarify the risk factors associated with the presence of *Campylobacter* in food animal and food animal products.

CLINICAL EXPRESSION OF *CAMPYLOBACTER* INFECTION IN HUMANS AND ANIMALS

In Humans. *Campylobacter jejuni* and *Campylobacter coli* have been recognized as important etiologic agents of gastrointestinal infection since the 1970s (Nachamkin, et al., 2000). In developed countries, typical clinical feature of *Campylobacter spp.* infection include acute, self-limiting gastroenteritis, characterized by diarrhea, fever and abdominal cramps, with a 24 to 72 hour incubation period (Allos, 2001). Diarrhea is initially watery, which may last more than two weeks in travellers's diarrhea (Gallardo, et al., 1998), or may become bloody as a result of diffuse inflammatory colitis and enteritis (Blaser, 1997). An important post-infection sequela of *Campylobacter jejuni* infection is Guillain-Barré syndrome (GBS), an acute demyelinating disease of the peripheral nervous system that results in a flaccid paralysis (Acheson, 2001; Altekruse, et al., 1998).

In developing countries, the clinical picture of *Campylobacter* infection is characterized by a milder form of gastroenteritis (Oberhelman and Taylor, 2000), and symptomatic infection is uncommon in adults (Blaser, 1997). Severe diarrhea requiring hospitalization is usually the result of co-infection with other virulent bacteria or viruses (Varavithya, et al., 1990). The severity of clinical symptoms and fecal excretion in children was inversely related to age (Taylor, et al., 1993). Although the severity of *Campylobacter* infection in adults was different between developed and developing countries, the clinical symptoms of infection in adults resulting from infection in developing countries was similar to those in developed countries (Oberhelman and Taylor, 2000).

In Food Animals. In cattle, *C. fetus* subsp. *venerealis* is the most frequently encountered *Campylobacter* in bovine abortion or infertility, while *C. fetus* subsp. *fetus* causes sporadic abortion in cattle and enzootic abortion in sheep (Garcia, et al., 1983). Bovine venereal campylobacteriosis is a chronic infection of the female genital tract, characterized by mild endometritis and transient infertility (Garcia, et al., 1983). Infection in bulls is accompanied by neither histological changes nor modifications in the characteristic of semen (Garcia, et al., 1983). *C. fetus* subsp. fetus infection in ewes resulting in abortion commonly in the last six week of gestation (Garcia, et al., 1983).

The role of *C. jejuni* as a primary pathogen in farm animals is uncertain. *C. jejuni* can be found in feces of diarrheic and healthy calves (Radostits, et al., 2000) and piglets (Steinhauserova, et al., 2001), but both *C. jejuni* and *C. coli* can cause a mild self-limiting enteritis and bacteremia when inoculated orally to newborn calves (Radostits, et al., 2000). In cows, there has been a single report of mastitis caused by *C. jejuni* (Morgan, et al., 1985).

ANTIMICROBIAL RESISTANCE IN *CAMPYLOBACTER*

Antimicrobial resistance has become a major public health concern in both developed and developing countries in recent years (Isenbarger, et al., 2002; Witte, 1998). Resistance to antimicrobial agents in respiratory and enteric bacteria poses the risk of treatment failure and increased cost of treatment (Vasallo, et al., 1998). *Campylobacter* with resistance to antimicrobial agents have been reported in both

developed and developing countries (Table 1-1), and the situation seems to deteriorate more rapidly in developing countries, where there is widespread and uncontrolled use of antibiotics (Hart and Kariuki, 1998). Fluoroquinolones and macrolides are the antimicrobial agents of choice for empirical treatment of gastroenteritis, and therefore resistance to these classes of antimicrobial agents are of paramount concern in *Campylobacter* (Engberg, et al., 2001).

Mechanisms of resistance to antimicrobial agents in *Campylobacter spp.* Resistance to aminoglycosides is normally mediated by enzymes that modify the drugs (Aarestrup and Engberg, 2001). Mechanism of resistance to chloramphenicol depends on the ability to produce enzymes that block binding of the drug to the ribosome, which is the target site of action (Aarestrup and Engberg, 2001). In *C.jejuni* and *C.coli*, resistance to tetracycline was found to be located on a transmissible plasmid encoding ribosomal protection gene (Aarestrup and Engberg, 2001). Macrolide resistance in *C.jejuni* is chromosomally mediated through mutation of the 23S rRNA gene (Engberg, et al., 2001). Mechanism of fluoroquinolone resistance in *C.jejuni* was also found to be chromosomally mediated through mutation of the *gyrA* gene (Wang, et al., 1993) and *parC* gene (Gibreel, et al., 1998). Resistance to azithromycin was found to be correlated with resistance to quinolones (Isenbarger, et al., 2002). Resistance to more than one group of antimicrobial agents in *C.jejuni* may be the result of efflux mechanism (Charvalos, et al., 1995).

Antimicrobial susceptibility testing. There are several methods currently being used for antimicrobial susceptibility testing of *Campylobacter*. Agar dilution,

commonly used in Europe, gives qualitative results but requires a high level of standardization for comparison (Caprioli, et al., 2000). Broth dilution provides quantitative data and is highly reproducible, but is more expensive than agar dilution (Caprioli, et al., 2000). The E-test (AB Biodisk, Culver city,CA) was found to give results comparable to the agar dilution test (Huang, et al., 1992), but is also expensive. Various genetic methods for assessing antimicrobial resistance are also available (Cockerill III, 1999). Comparisons of the results of antimicrobial susceptibility testing should be made with care, since different methods from the same laboratory or similar methods in different laboratories may yield different results (Caprioli, et al., 2000). For surveillance, quantitative data may be more desirable, since it can detect trends that indicate reduced susceptibility, and interventions can be implemented before high levels of resistance have developed (Walker and Thornsberry, 1998).

Antimicrobial resistance in human isolates. Increasing prevalence of quinolone-resistant *Campylobacter spp.* has been observed in the Netherlands since the early 1990's (Endtz, et al., 1990; Talsma, et al., 1999), where resistance to fluoroquinolones increased from 11% in 1994 to 29% in 1997 (Talsma, et al., 1999). Quinolone-resistant *Campylobacter* have recently been reported in many other countries, including Canada (Gaudreau and Gilbert, 1998) and Spain (Prats, et al., 2000). In the U.S., *Campylobacter* with resistance to nalidixic acid increased from 1.3% in 1992 to 10.2% in 1998 (Smith, et al., 1999).

Resistance to erythromycin appeared in the Netherlands in the 1990s (Talsma, et al., 1999). Resistance to macrolides has been reported to be as high as 90% in Spain

(Mirelis, et al., 1999), but trends over time for erythromycin resistance show stable and low rates in Japan, Canada and Finland (Engberg, et al., 2001). Resistance to macrolides was found to be more prevalent in *C. coli* than *C. jejuni* (Mirelis, et al., 1999; Saenz, et al., 2000).

There are several risk factors associated with the isolation of antimicrobial-resistant *Campylobacter.* Isolation of *Campylobacter* with resistance to quinolones was found to be associated with foreign travel and use of quinolone prior to isolation (Smith, et al., 1999). Exposure to fluoroquinolones in both humans and animals was found to induce resistance in *Campylobacter* (McDermott, et al., 2002; Segreti, et al., 1992; Wretlind, et al., 1992). The frequency of isolation also showed seasonal patterns with lower levels of resistant isolates in the summer months, when the overall incidence of *Campylobacter* isolation was higher (Talsma, et al., 1999). Consumption of contaminated foods is also a source of resistant bacteria: it has been estimated that, in the worse case scenario where prevalence of resistance *Campylobacter* is as high as 84%, 3-4 people per year in US will die as a result of infection with fluoroquinolone resistant *Campylobacter spp.* from beef consumption (Anderson, et al., 2001).

Antimicrobial resistance in human *Campylobacter* isolates has been found in developing countries. In Kenya, 51% of diarrhea patient had isolates of bacteria that were not susceptible to antimicrobial treatment, and 24% of *Campylobacter* was resistance to nalidixic acid (Shapiro, et al., 2001). In Thailand, resistance to azithromycin remained low (6%) (Isenbarger, et al., 2002) and therefore may be used empirically to treat gastroenteritis (Kuschner, et al., 1995). Similar to developed countries, resistance to macrolides was found to be more prevalent in *C. coli* than *C. jejuni* in Taiwan (Li, et al.,

1998), with resistance to erythromycin in 50% of *C. coli* isolated from human patients (Li, et al., 1998). In Thailand where fluoroquinolones are widely used in broiler farms to control respiratory disease, the prevalence of quinolone resistance *Campylobacter spp.* was found to increase from 0% in 1987 to 84% in 1995 (Hoge et al., 1998).

Antimicrobial resistance in isolates from food animals. The resistance to fluoroquinolones in food animal was observed in the Netherland since early 1990's (Endtz, et al., 1990). Since then, there have been several reports of antimicrobial resistance in *Campylobacter spp.* isolated from food animals in both developed and developing countries (Table 1-2). High level of resistance to fluoroquinolones and tetracycline were reported in the Netherland (Jacob-Reitsma, et al., 1994), Spain (Saenz, et al., 2000), Ireland (Lucey, et al., 2000) and Taiwan (Li, et al., 1998). Resistance to tetracycline was much higher in Taiwan compared to other countries in Europe (Table 1-2). Resistance to gentamycin was relatively low in Spain (Saenz, et al., 2000), Ireland (Lucey, et al., 2000) and Taiwan (Li, et al., 1998). Only resistance to chloramphenicol was found in *Campylobacter spp.* isolated from chickens in Switzerland (Frei, et al., 2001).

Link between resistance in food animal isolates to resistance in human isolates. There has been speculation that the use of antimicrobial agents, such as quinolones, in food animals resulted in increasing the prevalence of quinolone resistance in *Campylobacter spp.* from humans (Endtz, et al., 1991; Gaunt and Piddock, 1996; Piddock, 1996) followed the approval of fluoroquinolone use in food animals. There seems to be temporal relationship between use of fluoroquinolones in animals and the

finding of resistant *Campylobacter* in humans (Engberg, et al., 2001). In Spain, the proportion of human isolates of *Campylobacter* resistant to quinolones was found to be 72%, while 99% of *Campylobacter* isolated from broilers and pigs were resistant to quinolones (Saenz, et al., 2000). In Taiwan, 52% of *C. jejuni* isolated from human patients were resistance to ciprofloxacin, while 92% of isolates from chickens were resistant to the same agent (Li, et al., 1998). Since *C. coli* was the the most common species of *Campylobacter* isolated from pigs (Steinhauserova, et al., 2001), resistance to macrolides in human isolates may reflect the use of macrolides in pork production in Taiwan. In Denmark, it was shown that proportion of *Enterocooci* with resistance to avoparcin, tylosin, erythromycin, Virginiamycin and avilamycin reduced after those agents were banned from the market (Aarestrup, et al., 2001). This observation indicated significant association between antimicrobial use as growth promoter in food animal and prevalence of resistance bacteria. Similar phenomenon may be observed in *Campylobacter spp.*, which should be confirmed.

In summary, resistance was observed at high levels in food animals in both developed and developing countries. Studies suggested an association between antimicrobial use in food animals and the development of resistance in human isolates in developed countries. Traveling to developing countries has also been related to isolation of resistant *Campylobacter.* However, there have been no reports of risk factors for the development of antimicrobial resistance in developing countries. In most developing countries, while antimicrobial use is usually less regulated than developed countries, economic necessity may limit the use of newer, more expensive antimicrobial agents. An

epidemiological study of risk factors associated with the development of antimicrobial resistance in developing countries will be valuable, in order to prolong the usefulness of the available antimicrobial agents for treatment of important human pathogens.

Table 1-1 Prevalence of antimicrobial resistance in *Campylobacter spp.* from humans.

Country	Year	Method	CIP/FQ[1]	ERY/M[1]	CHL[1]	GEN[1]	TET[1]	Ref.
Netherland	1994-1997	Agar diffusion	28.8	0.3			16.1	(Talsma, et al., 1999)
Spain	1995-1998	Disk diffusion	81	5		1	72	(Prats, et al., 2000)
Spain	1979-1996	Disk diffusion	54	7		0	0	(Pigrau, et al., 1997)
Spain	1997-1998	Disk diffusion	75	3.2		0.4		(Saenz, et al., 2000)
Spain	1992-1993	Broth dilution or disk diffusion	30.4			0	44.3	(Velazquez, et al., 1995)
Spain	1999	NA	81-90	4-90		1-16	70-97	(Mirelis, et al., 1999)
Ireland	1996-1998	Agar dilution	17	3	3	0	14	(Lucey, et al., 2000)
UK	1997	Agar dilution	11.7	1.7	5.4	0.1	29.6	(Thwaites and Frost, 1999)
Canada	1995-1997	Agar dilution	12.7-13.9	0			55.7	(Gaudreau and Gilbert, 1998)
US	1998	E-test	10.2					(Smith, et al., 1999)
Kenya	1997-1998	E-test	24	5	5		5	(Shapiro, et al., 2001)
Nigeria	1998	Disk diffusion	-	16.7	33.3	0	33.3	(Smith, et al., 1999)
Egypt	1986-1993	Disk diffusion	24-40	9-10	0-3	3-7	6-24	(Wasfy, et al., 2000)
India	1994	Disk diffusion	4.4	2.2			6.7	(Prasad, et al., 1994)
India	1996	Disk diffusion			100			(Chuma, et al., 2001)
Taiwan	1998	E-test	52-85	10-50		1-15	85-95	(Li, et al., 1998)
Taiwan	1994	Broth dilution					91	(Lee, et al., 1994)
Thailand	1995	Agar dilution	84	7				(Hoge, et al., 1998)
Thailand	1994	Broth/agar dilution	69-79	0-31				(Murphy Jr, et al., 1996)
Thailand	1995-1999	Agar dilution	73-77	6				(Isenbarger, et al., 2002)

[1] AMP-ampicillin, CHL-chloramphenicol, CIP/FQ-ciprofloxacin or other fluoroquinolones, ERY/M-erythromycin or other macrolides, GEN-gentamycin, TET – tetracycline

[2] NA - not available

Table 1-2 Prevalence of antimicrobial resistance in *Campylobacter spp.* from food animals, food, and water.

Source	Country	Year	Method	CIP/FQ[1]	ERY/M[1]	CHL[1]	GEN[1]	TET[1]	Ref.
Animal	Netherland	1994	Disk diffusion	29.3-34.7	4			16	(Jacob-Reitsma, et al., 1994)
	Spain	1997-1998	Disk diffusion	98.7	1.7	0	11.9	31.8	(Saenz, et al., 2000)
	Ireland	1996-1998	Agar dilution	16	9	6	3	19	(Lucey, et al., 2000)
	Swiss	2001	E-test	0	0	8	0	0	(Frei, et al., 2001)
	US	1997	E-test	14					(Smith, et al., 1999)
	Nigeria	1998	Disk diffusion		41.4	13.5	3.9	27.7	(Smith, et al., 1999)
	India	1994	Disk diffusion	0	0			13.3	(Prasad, et al., 1994)
	India	1996	Disk diffusion			60.34		27.58	(Das, et al., 1996)
	Taiwan	1998	E-test	91-92	17-83		0	83-96	(Li, et al., 1998)
	Taiwan	1994	Broth dilution					44	(Lee, et al., 1994)
	Japan	1995-1999	Broth dilution	32.4	0				(Chuma, et al., 2001)
Food	Spain	1997-1998	Disk diffusion	74.7	17.5	2.4	4.9	65	(Saenz, et al., 2000)
Water	Netherland	1995	Disk diffusion	16-21	9-25			19-50	(Koenraad, et al., 1995)

[1] CHL-chloramphenicol, CIP/FQ-ciprofloxacin or other fluoroquinolones, ERY/M-erythromycin or other macrolides, GEN-gentamycin, TET – tetracycline

REFERENCES

Aarestrup, F., and J. Engberg. 2001. Antimicrobial resistance of thermophilic *Campylobacter*. Veterinary research. 32:311-321.

Aarestrup, F., A. Seyfarth, H. Emborg, K. Pedersen, R. Hendriksen, and F. Bager. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurence of antimicrobial resistance in fecal *Enterococci* from food animals in Denmark. Antimicrobial agents and chemotherapy. 45(7):2054-2059.

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Altekruse, S., D. Swerdlow, and N. Stern. 1998. *Campylobacter jejuni*. Veterinary clinics of North America: Food animal practice. 14(1):31-40.

Anderson, S. A., Y. Woo, and L. M. Crawford. 2001. Risk assessment of the impact on human health of resistant *Campylobacter jejuni* from fluoroquinolone use in beef cattle. Food control. 12(1):13-25.

Atanassova, V., and C. Ring. 1999. Prevalence of Campylobacter spp. in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Berndtson, E., M. Danialsson-Tham, and A. Engvall. 1996. Campylobacter incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. International journal of food microbiology. 32:35-47.

Berndtson, E., U. Emanuelson, A. Engvall, and M. L. Danielsson-Tham. 1996. A 1-year epidemiological study of *Campylobacter* in 18 Swedish chicken farms. Preventive veterinary medicine. 26:167-185.

Berrang, M., and J. Dickens. 2000. Presence and level of *Campylobacter spp.* on broiler carcasses throughout the processing plant. Journal of applied poultry research. 9:43-47.

Berrang, M., S. Ladely, and R. Buhr. 2001. Presence and level of *Campyobacter*, coliforms, *Escherichia coli*, and total aerobacteria recovered from broiler parts with and without skin. Journal of food protection. 64(2):184-188.

Blaser, M. 1997. Epidemiology and clinical features of *Campylobacter jejuni* infections. The journal of infectious diseases. 176(suppl 2):s103-s105.

Busato, A., T. Lentze, D. Hofer, A. Burnens, B. Hentrich, and C. Gaillard. 1998. A case-

control study of potential enteric pathogens for calves raised in cow-calf herds. Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 45:519-528.

Caprioli, A., L. Busani, J. Martel, and R. Helmuth. 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. International Journal of antimicrobial agents. 14:295-301.

Cawthraw, S., L. Lind, B. Kaijser, and D. Newell. 2000. Antibodies, directed towards *Campylobacter jejuni* antigens, in sera from poultry abattoir workers. Clinical and experimental immunology. 122:55-60.

Charvalos, E., Y. Tselentis, M. Hamzehpour, T. Kohler, and J. Pechere. 1995. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrobial agents and chemotherapy. 39(9):2019-2022.

Chuma, T., S. Hashimoto, and K. Okamoto. 2000. Detection of thermophilic *Campylobacter* from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with *Campylobacter*. Journal of veterinary medical science. 62(12):1291-1295.

Chuma, T., T. Ikeda, T. Maeda, H. Niwa, and K. Okamoto. 2001. Antimicrobial susceptibilities of *Campylobacter* strains isolated from broilers in southern part of Japan from 1995-1999. Journal of veterinary medical science. 63(9):1027-1029.

Chuma, T., K. Makino, K. Okamoto, and H. Yugi. 1997. Analysis of distribution of *Campylobacter jejuni* and *Campylobacter coli* in broiler by using restriction fragment polymorphism of flaggelin gene. Journal of veterinary medical scienes. 59(11):1011-1015.

Cockerill III, F. 1999. Genetic methods for assessing antimicrobial resistance. Antimicrobial agents and chemotherapy. 43(2):199-212.

Craven, S., N. Stern, E. Line, J. Bailey, N. Cox, and P. Fedorka-Cray. 2000. Determination of the incidence of *Salmonella spp.*, *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal dropping. Avian diseases. 44:715-720.

Das, S. C., G. B. Nair, S. G. Mullickk, G. Biswas, A. Sikdar, and D. Bhatacharya. 1996. Study on in vitro antimicrobial sensitivity of *Campylobacter* species of animal and human origin. Indian journal of animal health. 35(2):193-196.

Deming, M., R. Tauxe, P. Blake, S. Dixon, B. Fowler, S. Jones, E. Lockamy, C. Patton, and R. Sikes. 1987. *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. American journal of epidemiology. 126(3):526-534.

Duffy, E., K. Belk, J. Sofos, G. Bellinger, A. Pape, and G. Smith. 2001. Extent of microbial contamination in United States pork retail products. Journal of food protection. 64(2):172-178.

Dufrenne, J., W. Ritmeester, E. Delfgou-van-Asch, F. Leusden, and R. Jonge. 2001. Quantification of contamination of chicken and chicken products in the Netherlands with *Salmonella* and *Campylobacter*. Journal of food protection. 64(4):538-541.

Effler, P., M. Ieong, A. Kimura, M. Nakata, R. Burr, E. Cremer, and L. Slutsker. 2001. Sporadic *Campylobacter jejuni* infections in Hawaii: Association with prior antibiotic use and commercially prepared chicken. The journal of infectious diseases. 183:1152-1155.

Endtz, H. P., R. P. Mouton, T. D. Reyden, G. J. Ruijs, M. Biever, and B. Klingeren. 1990. Fluoroquinolone resistance in *Campylobacter spp.* isolated from human stool and poultry product. The Lancet. 335:787.

Endtz, H. P., G. J. Rujis, B. Klingeren, W. H. Jansen, T. Reyden, and R. P. Mouton. 1991. Quinolone resistance in *campylobacter* isilated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. Journal of antimicrobial chemotherapy. 27:199-208.

Engberg, J., F. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C.coli*: resistance mechanisms and trends in human isolates. Emerging infectious diseases. 7(1):24-34.

Evans, S. J., and A. R. Sayers. 2000. A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. Preventive veterinary medicine. 46:209-223.

Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones. 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. Applied and environmental microbiology. 67(4):1429-1436.

Frei, A., D. Goldenberger, and M. Teuber. 2001. Antimicrobial susceptibility of intestinal bacteria from swiss poultry flocks before the band of antimicrobial growth promotors. Systemic and applied microbiology. 24:116-121.

Friedman, C., J. Neimann, H. Wegener, and R. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121-138. *In* I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Gallardo, F., J. Gascon, J. Ruiz, M. Corachan, T. Anta, and J. Vila. 1998. *Campylobacter jejuni* as a cause of traveler's diarrhoea: clinical features and antimicrobial susceptibility. Journal of travel medicine. 5:23-26.

Garcia, M., M. Eaglesome, and C. Rigby. 1983. *Campylobacter* important in veterinary medicine. Veterinary bulletin. 53(9):793-818.

Gaudio, P., P. Echeverria, C. Hoge, J. Pitarangsri, and P. Goff. 1996. Diarrhea among expatriate residents in Thailand: correlation between reduced *Campylobacter* prevalence and longer duration of stay. Journal of traveller medicine. 3:77-79.

Gaudreau, C., and H. Gilbert. 1998. Antimicrobial resistance of clinical strains of *Campylobacter jejuni* subsp. *jejuni* isolated from 1985 to 1997 in Quebec, Canada. Antimicrobial agents and chemotherapy. 42(8):2106-2108.

Gaunt, P. N., and L. J. V. Piddock. 1996. Ciprofloxacin resistant *Campylobacter spp.* in humans: an epidemiological and laboratory study. Journal of antimicrobial chemotherapy. 37:747-757.

Genigeorgis, C., M. Hassuneh, and P. Collins. 1986. *Campylobacter jejuni* infection on poultry farms and its effect on poultry meat contamination during slaughtering. Journal of food protection. 49(11):895-903.

Gibreel, A., E. Sjogren, B. Kaijser, B. Wretlind, and O. Skold. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. Antimicrobial agents and chemotherapy. 42(12):3276-3278.

Hald, B., E. Rattenborg, and M. Madsen. 2001. Role of batch depletion of broiler houses on the occurence of *Campylobacter spp.* in chicken flocks. Letters in applied microbiology. 32:253-256.

Hart, C., and S. Kariuki. 1998. Antimicrobial resistance in developing countries. British medical journal. 317:647-650.

Harvey, R., C. Young, R. Anderson, R. Droleskey, K. Genovese, L. Egan, and D. Nisbet. 2000. Diminution of *Campylobacter* colonization in neonatal pigs reared off-sow. Journal of food protection. 63(10):1430-1432.

Harvey, R. B., C. R. Young, R. L. Ziprin, M. E. Hume, K. J. Genovese, R. C. Anderson, R. E. Drolesky, L. H. Stanker, and D. J. Nisbet. 1999. Prevalence of *Campylobacter spp.* isolated from the intestinal tract of pigs raised in an integrated swine production system. Journal of the american veterinary medical association. 215(11):1601-1604.

Heuer, O., K. Pedersen, J. Andersen, and M. Madsen. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Letters in applied microbiology. 33:269-274.

Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsri, and P. Echeverria. 1998. Trends in

antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clinical infectious diseases. 26:341-345.

Hopkins, R., and A. Scott. 1983. Handling raw chicken as a source for sporadic *Campylobacter jejuni* infections. The journal of infectious diseases. 148(4):770.

Huang, M., C. Baker, S. Banerjee, and F. Tenover. 1992. Acuracy of the E test for determining antimicrobial susceptibilities of *Staphylococci*, *Enterococci*, *Campylobacter jejuni*, and gram-negative bacteria resistant to antimicrobial agents. Journal of clinical microbiology. 30(12):3243-3248.

Isenbarger, D., C. Hoge, A. Srijan, C. Pitarangsi, N. Vithayasai, L. Bodhidatta, K. Hickey, and P. Cam. 2002. Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996-1999. Emerging infectious diseases. 8(2):175-180.

Jacob-Reitsma, W. 1995. *Campylobacter* bacteria in breeder flocks. Avian diseases. 39:355-359.

Jacob-Reitsma, W. 1997. Aspects of epidemiology of *Campylobacer* in poultry. Veterinary quarterly. 19(3).

Jacob-Reitsma, W., N. Bolder, and R. Mulder. 1994. Cecal carriage of *Campylobacter* and *Salmonella* in Dutch broiler flocks at slaughter: A one-year study. Poultry science. 73:1260-1266.

Jacob-Reitsma, W., P. Koenraad, N. Bolder, and R. Mulder. 1994. In vitro susceptibility of *Campylobacter* and *Salmonella* isolates from broilers to quinolones, ampicillin, tetracycline, and erythromycin. Veterinary quarterly. 16(4):206-208.

Jacob-Reitsma, W., A. Van de Giessen, N. Bolder, and R. Mulder. 1995. Epidemiology of *Campylobacter spp.* at two dutch broiler farm. Epidemiology and infections. 114:413-421.

Jones, F., R. Axtell, D. Rives, S. Scheideler, F. Tarver, R. Walker, and M. Wineland. 1991. A survey of *Campylobacter jejuni* Contamination in modern broiler production and processing system. Journal of food protection. 54(4):259-262.

Kalman, M., E. Szollosi, B. Czermann, M. Zimanyi, S. Szekeres, and M. Kalman. 2000. Milkborne *Campylobacter* infection in Hungary. Journal of food protection. 63(10):1426-1429.

Kapperud, G., E. Skjerve, N. Bean, S. Ostroff, and J. Lassen. 1992. Risk factors for sporadic *Campylobacter* infections : result of a case-control study in Southeatern Norway. Journal of clinical microbiology. 30(12):3117-3121.

Kazwala, R., J. Collins, R. Crinion, and H. O'Mahony. 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commercial poultry production. The veterinary record. 126:305-306.

Koenraad, P. M. F. J., W. F. Jacob-Reitsma, T. V. D. Laan, R. R. Reumer, and F. M. Rombouts. 1995. Antibiotic susceptibility of *Campylobacter* isolates from sewage and poultry abattoir drain water. Epidemiology and infection. 115:475-483.

Korsak, N., G. Daube, Y. Ghafir, A. Chahed, S. Jolly, and H. Vindevogel. 1998. An efficient sampling technique used to detect four foodborne pathogens on pork and beef carcasses in nine Belgian abattoirs. Journal of food protection. 61(5):535-541.

Kuschner, R. A., F. A. Trofa, R. J. Thomas, C. W. Hoge, C. Pitarangsi, S. Amato, R. P. Olafson, P. Echeveria, J. C. Sadoff, and D. N. Taylor. 1995. Use of Azithromicin for the treatment of *Campylobacter* enteritis in travellers to Thailand, an area where Ciprofloxacin resistance is prevalent. Clinical infectious diseases. 21:536-541.

Lee, C. Y., C. L. Tai, S. C. Lin, and Y. T. Chen. 1994. Occurence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. International journal of food microbiology. 24:161-170.

Lehner, A., C. Schneck, G. Feierl, P. Plees, A. Deutz, E. Brandl, and M. Wagner. 2000. Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of *Campylobacter jejuni* in an Austrian youth centre. Epidemiology and infections. 125:13-16.

Li, C. C., C. H. Chiu, J. L. Wu, Y. C. Huang, and T. Y. Lin. 1998. Antimicrobial susceptibilities of *Campylobacter jejuni* and coli by using E-test in Taiwan. Scandinavian journal of infectious disease. 30:39-42.

Lucey, B., C. Feurer, P. Greer, P. Moloney, B. Cryan, and S. Fanning. 2000. Antimicrobial resistance profiling and DNA Amplification fingerprinting (DAF) of thermophilic *Campylobacter spp.* in human, poultry and porcine samples from the Cork region of Ireland. Journal of applied microbiology. 89:727-734.

McDermott, P., S. Bodeis, L. English, D. White, R. Walker, S. Zhao, S. Simjee, and D. Wagner. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. The journal of infectious diseases. 185:837-840.

Mirelis, B., T. Llovet, C. Munoz, F. Navarro, and G. Prats. 1999. Resistance of *Salmonella* and *Campylobacter* species to antimicrobial agents. European journal of clinical microbiology and infectious diseases. 18:312.

Morgan, G., P. Chadwick, K. Lander, and K. Gill. 1985. *Campylobacter jejuni* mastitis in

a cow: a zoonosis related incident. The veterinary record. 116(4):111.

Murphy Jr, G., P. Echeverria, L. Jackson, M. Arness, C. Lebron, and J. Pitarangsi. 1996. Ciprofloxacin and azithromycin resistant *Campylobacter* causing traveller's diarrhea in US troops deployed to Thailand in 1994. Clinical infectious diseases. 22:868-869.

Nachamkin, I., J. Engberg, and F. M. Aaestrup. 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. *In* I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Newell, D., J. Shreeve, M. Toszeghy, G. Dominigue, S. Bull, T. Humphrey, and G. Mean. 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. Applied and environmental microbiology. 67(6):2636-2640.

Oberhelman, R., and D. Taylor. 2000. *Campylobacter* infections in developing countries, p. 139-153. *In* I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Olsen, S., G. Hansen, L. Bartlett, C. Fitzgerald, A. Sonder, R. Manjrekar, T. Riggs, J. Kim, R. Flahart, G. Pezzino, and D. Swerdlow. 2001. An outbreak of *Campylobacter jejuni* infections associated with food handler contamination: The use of pulse-field gel electrophoresis. The journal of infectious diseases. 183:164-167.

Osano, O., and S. Arimi. 1999. Retail poultry and beef as sources of *Campylobacter jejuni*. East african medical journal. 76(3):141-143.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by water borne *Campylobacter jejuni*. Applied and environmental microbiology. 59(4):987-996.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Pearson, A. D., M. H. Greenwood, R. K. A. Feltham, T. D. Healing, J. Donaldson, D. M. Jones, and R. R. Colwell. 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: Intermittent common source, vertical transmission, and amplification by flock propagation. Applied and environmental microbiology. 62(12):4614-4620.

Petersen, L., E. Nielsen, and S. On. 2001. Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. Veterinary microbiology. 82:141-154.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Piddock, L. J. V. 1996. Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic resistant bacteria that infect man and compromise antimicrobial chemotherapy. Journal of antimicrobial chemotherapy. 38(1):1-3.

Pigrau, C., R. Bartolome, B. Almirante, A. M. Planes, J. Gavalda, and A. Pahissa. 1997. Bacteremia due to *Campylobacter* species: clinical findings and antimicrobial susceptibility patterns. Clinical infectious diseases. 25:1414-1420.

Poocharoen, L., and C. Bruin. 1986. *Campylobacter jejuni* in hospitalized children with diarrhoea in Chiang Mai, Thailand. Southeast Asian journal of tropical medicine and public health. 17(1):53-58.

Prasad, K. N., S. K. Mathur, T. N. Dhole, and A. Ayyagari. 1994. Antimicrobial susceptibility and plasmid analysis of *Campylobacter jejuni* isolated from diarrhoeal patients and healthy chickens in Northern India. journal of diarrhoeal disease research. 12(4):270-273.

Prats, G., B. Mirelis, T. Llovet, C. Munoz, E. Miro, and F. Navarro. 2000. Antibiotic resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona. Antimicrobial agents and chemotherapy. 44(5):1140-1145.

Quinn, P., M. E. Carter, B. A. Markey, and G. R. Carter. 1994. Clinical veterinary microbiology. Wolfe publishing, London.

Radostits, O., C. Gay, D. Blood, and K. Hinchcliff. 2000. Veterinary Medicine, 9 ed. W.B.Saunders, London.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Refregier-Petton, J., N. Rose, M. Denis, and G. Salvat. 2001. Risk factors for *Campylobacter spp.* contamination in French broiler-chicken flocks at the end of the rearing period. Preventive veterinary medicine. 50:89-100.

Rivoal, K., M. Denis, G. Salvat, P. Colin, and G. Ermel. 1999. Molecular characterization of the diversity of *Campylobacter spp.* isolates collected from a poultry slaughterhouse: analysis of cross contamination. Letters in applied microbiology. 29:370-374.

Saeed, A., N. Harris, and R. DiGiacomo. 1993. The role of exposure to animals in the etiology of *Campylobacter jejuni/coli* enteritis. American journal of epidemiology. 137(1):108-114.

Saenz, Y., M. Zarazaga, M. Lantero, M. Gastanares, F. Bacuero, and C. Torres. 2000. Antibiotic resistance in *Campylobacter* strains isolated from animals, foods and human in Spain in 1997-1998. Antimicrobial agents and chemotherapy. 44(2):267-271.

Schorr, D., H. Schmid, H. Rieder, A. Baumgartner, H. Vorkauf, and A. Burnens. 1994. Risk factors for *Campylobacter* enteritis in Switzerland. Zbl. Hyg. 196:327-337.

Segreti, J., T. D. Gootz, L. J. Goodman, G. W. Parkhurst, J. P. Quinn, B. A. Martin, and L. J. Trenholme. 1992. High-level quinolone resistant in clinical isolates of *Campylobacter jejuni*. The journal of infectious diseases. 165:667-670.

Shapiro, R., L. Kumar, P. Phillips-Howard, J. Wells, P. Adcock, J. Brooks, M. Ackers, J. Ochieng, E. Mintz, S. Wahlquist, P. Waiyaki, and L. Slutsker. 2001. Antimicrobial resistant bacterial diarrhea in rural western Kenya. The journal of infectious diseases. 183(1701-1704).

Shih, D. Y. 2000. Isolation and identification of enteropathogenic *Campylobacter spp.* from chicken samples in Taipei. Journal of food protection. 63(1):304-308.

Simango, C., and G. Rukure. 1991. Potential sources of *Campylobacter* species in the home of farm workers in Zimbabwe. Journal of tropical medicine and hygiene. 94:388-392.

Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, and M. T. Osterholm. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. The new england journal of medicine. 340(20):1525-32.

Smith, S. I., T. I. Sansa, and A. O. Coker. 1999. Antibiotic susceptibility patterns and beta-lactamase production of animal and human isolates of *Campylobacter* in Lagos, Nigeria. Verlag der Zeitschrift fur naturforschung. 54c:583-586.

Stanley, K., J. Wallace, J. Currie, P. Diggle, and K. Jones. 1998. The seasonal variation of thermophilic *campylobacters* in beef cattle, dairy cattle and calves. Journal of applied microbiology. 85:472-480.

Steinhauserova, I., K. Fojtikova, and J. Klimes. 2001. The incidence and PCR detection of *Campylobacter upsaliensis* in dogs and cats. Letters in applied microbiology. 31:209-212.

Steinhauserova, I., K. Fojtikova, and J. Matiasovic. 2001. Subtyping of *Campylobacter*

spp. strains and the incidence in piglets. Acta veterinaria Brno. 70:197-201.

Stern, N., S. Green, N. Thaker, D. Krout, and J. Chiu. 1984. Recovery of *Campylobacter jejuni* from fresh and frozen meat and poultry collected at slaughter. Journal of food protection. 47(5):372-374.

Stern, N., M. Hernandez, L. Blankenship, K. Deibel, S. Doores, M. Doyle, H. NG, M. Pierson, J. Sofos, W. Sveum, and D. Westhoff. 1985. Prevalence and distribution of *Campylobacter jejuni* and *Campylobacter coli* in retail meats. Journal of food protection. 48(7):595-599.

Stern, N., K. Hiett, N. Cox, G. Alfredsson, K. Kristinsson, and J. Line. 2000. Recent developments pertaining to *Campylobacter*. Irish journal of agricultural and food research. 39:183-187.

Stern, N. J., M. R. S. Clavero, J. S. Bailey, N. A. Cox, and M. C. Robach. 1995. *Campylobacter spp.* in broilers on the farm and after transport. Poultry science. 74:937-941.

Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous *campylobacter* infection: A swedish case-control study. Epidemiology and infections. 125:269-275.

Talsma, E., W. G. Goettsch, H. L. J. Nieste, P. M. Schrijnemakers, and M. J. W. Sprenger. 1999. Resistance in *Campylobacter* species: increased resistance to fluoroquinolones and seasonal variation. Clinical infectious diseases. 29:845-848.

Tay, S. T., S. D. Puthucheary, S. Devi, and I. Kautner. 1995. Characterisation of *Campylobacters* from Malaysia. Singapore medical journal. 36:282-284.

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

Thwaites, R. T., and J. A. Frost. 1999. Drug resistance in *Campylobacter jejuni*, *C. coli* and *C. lari* isolated from humans in North West England and Wales, 1997. Journal of clinical pathology. 52:812-814.

Uyttendaele, M., P. D. Troy, and J. Debevere. 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and Listeria monocytogenes in poultry carcasses and different types of poultry products for sale on the Belgian retail market. Journal of food protection. 62(7):735-740.

Van de Giessen, A., S. I. Mazurier, W. Jacob-Reitsma, W. Jansen, P. Berkers, W. Ritmeester, and K. Wernars. 1992. Study on the Epidemiology and control of *Campylobacter jejuni* in poultry broiler flocks. Applied and environmental microbiology.

58(6):1913-1917.

Van de Giessen, A. W., B. P. M. Bloemberg, W. S. Ritmeester, and J. J. H. C. Tilburg. 1996. Epidemiological study on risk factors and risk reducing measures for *Campylobacter* infections in Dutch broiler flocks. Epidemiology and infection. 117:245-250.

Vandamme, P. 2000. Taxonomy of the family Campylobacteriaceae, p. 3-26. *In* I. Nachamkin and M. Blaser (ed.), *Campylobacter*, vol. 2. ASM press, Washington DC.

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Vasallo, F. J., P. M. Rabadan, L. Alcala, J. M. G. Lechuz, M. R. Creixems, and E. Bouza. 1998. Failure of ciprofloxacin therapy for invasive nontyphoidal *Salmonellosis*. Clinical infectious diseases. 26:535-536.

Velazquez, J., A. Jimenez, B. Chomon, and T. Villa. 1995. Incidence and transmission of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli*. Journal of antimicrobial chemotherapy. 35:173-178.

Walker, R. D., and C. Thornsberry. 1998. Decrease in antibiotic susceptibility or increase in resistance? Journal of antimicrobial chemotherapy. 41(1):1-4.

Wang, Y., W. M. Huang, and D. E. Taylor. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutation. Antimicrobial agents and chemotherapy. 37(3):457-463.

Wasfy, M., B. Oyofo, J. David, T. Ismail, A. El-Gendy, Z. Mohran, Y. Sultan, and L. Peruski. 2000. Isolation and antibiotic susceptibility of *Salmonella*, *Shigella* and *Campylobacter* from acute enteric infections in Egypt. Journal of health and population nutrition. 18(1):33-38.

Wassenaar, T., and D. Newell. 2000. Genotyping of *Campylobacter spp*. Applied environmental microbiology. 66(1):1-9.

Wedderkopp, A., E. Rattenborg, and M. Madsen. 2000. National surveillance of *Campylobacter* in broilers at slaughter in Denmark in 1998. Avian diseases. 44:993-999.

Weijtens, M. J. B. M., J. Plas, P. G. H. Bijker, H. A. P. Urling, D. Koster, J. G. Logtestijn, and J. H. J. Veld. 1997. The transmission of *Campylobacter* in piggeries; an epidemiological study. Journal of applied microbiology. 83:693-698.

Weijtens, M. J. B. M., R. D. Reinders, H. A. P. Urling, and J. Plas. 1999. *Campylobacter* infections in fattenning pigs; excretion pattern and genetic diversity. Journal of applied microbiology. 86:63-70.

Wesley, I., S. Wells, K. Harmon, A. Green, L. Schroeder-Tucker, M. Glover, and I. Siddique. 2000. Fecal shedding of *Campylobacter* and *Arcobacter spp.* in dairy cattle. Applied and environmental microbiology. 66(5):1994-2000.

Whyte, P., J. Collins, K. McGill, C. Monahan, and H. O'Mahony. 2001. The effect of transportation stress on excretion rates of *Campylobacters* in market-age broiler. Poultry sciences. 80:817-820.

Whyte, P., C. JD, K. McGill, C. Monahan, and H. O'Mahony. 2001. Distribution and prevalence of airborne microorganisms in three commercial poultry processing plants. Journal of food protection. 64(3):388-391.

Willis, W. L., and C. Murray. 1996. *Campylobacter jejuni* seasonal recovery observations of retail market broilers. Poultry science. 76:314-317.

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. Science. 279:996-7.

Wretlind, B., A. Stromberg, L. Ostlund, E. Sjogren, and B. Kaijser. 1992. Rapid emergence of quinolone resistance in *Campylobacter jejuni* in patient treated with Norfloxacin. Scandinavian journal of infectious diseases. 24:685-686.

Young, C., R. Harvey, R. Anderson, D. Nisbet, and L. Stanker. 2000. Enteric colonisation following natural exposure to *Campylobacter* in pigs. Research in veterinary science. 68(1):75-78.

CHAPTER 2

MOLECULAR TECHNIQUES IN EPIDEMIOLOGICAL STUDY OF *CAMPYLOBACTER SPP.* : A REVIEW

INTRODUCTION

Campylobacter spp. Have been recently recognized as an emerging food borne bacteria in many developed countries (Acheson, 2001; Pearson, et al., 2000; Phetsouvanh, et al., 1999; Tay, et al., 1995). *C. jejuni* and *C. coli* have been recognized as important etiologic agent of gastrointestinal infection since 1970 (Nachamkin, et al., 2000). Typical clinical features of *Campylobacter spp.* infection include acute, self-limiting gastroenteritis characterized by diarrhea, fever and abdominal cramps (Allos, 2001). The incubation period usually ranges between 24-72 hours. Diarrhea is initially watery then becomes bloody, which is a result of diffuse inflammatory colitis and enteritis (Blaser, 1997). In developing countries, symptomatic infection is uncommon in adult (Blaser, 1997). *C. jejuni* is also considered a cause of travelers' diarrhea resulting in watery diarrhea which last more than 14 days (Gallardo, et al., 1998). An important post-infection sequel of *C. jejuni* infection is Guillain-Barre syndrome (GBS), an acute demyelinating disease of the peripheral nervous system resulting in flaccid paralysis (Acheson, 2001; Altekruse, et al., 1998).

Campylobacter spp. are associated with both sporadic and outbreak infection. In the US, the incidence of *Campylobacter spp.* infection was found to be 20.1 cases per 100,000 population in 2000 (Acheson, 2001). Outbreaks of *Campylobacter spp.* are usually associated with raw milk (Altekruse, et al., 1998; Kalman, et al., 2000), whereas

sporadic illnesses are often associated with consumption of chickens (Deming, et al., 1987; Effler, et al., 2001).

In Southeast Asia, *Campylobacter spp.* infection was found predominantly in children less than 5 years old. Previously reported prevalences of *Campylobacter spp.* in this area range from 2.9% - 15% (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988; Varavithya, et al., 1990) in children, and up to 10% in adults (Gaudio, et al., 1996). In diarrheic children in Thailand, *Campylobacter spp.* was isolated as frequently as entero-toxogonic E.*coli* and Salmonella (Rasrinual, et al., 1988). There was no obvious seasonal pattern, and childrens with *Campylobacter spp.* are often co-infected with E.*coli*, Salmonella or Shigella (Poocharoen and Bruin, 1986).

Because of the growing concern over the public health significance of *Campylobacter spp.*, various molecular techniques have been developed and applied in epidemiological studies of *Campylobacter spp.* A review of genotyping techniques for *Campylobacter spp.* was recently published (Wassenaar and Newell, 2000). However, a review of the application of molecular techniques in epidemiological studies is not available. The objectives of this review, therefore, were to 1.) summarize available molecular techniques applicable to *Campylobacter spp.* and 2.) review the applications of molecular techniques in epidemiological studies of *Campylobacter spp.* A comparison of available techniques and suggestions for the application of molecular techniques in epidemiological studies of *Campylobacter spp.* will be discussed.

AVAILABLE MOLECULAR TECHNIQUES FOR EPIDEMIOLOGICAL STUDIES OF *CAMPYLOBACTER SPP.*

Molecular techniques for detection and identification of *Campylobacter spp.*

Campylobacter spp. are slow-growing, fastidious organisms, which prefer microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) (Nachamkin, et al., 2000). Most *Campylobacter* grow well at 37°C, but a group of thermophilic *Campylobacter*, such as *C. jejuni* and *C. coli*, also grow well at 42°C. Isolation and identification of *Campylobacter* usually begins by culture in selective enrichment media, which includes antibiotics to limit the growth of competitive organisms, and ingredients to neutralize the toxic effects of oxygen and light (Corry, et al., 1995). There is no single selective media able to recover all types of *Campylobacter spp.* (Moore and Murphy, 2000). Because of the strict conditions required for bacterial growth, the length of time required for culture (6 days) (Nachamkin, et al., 2000), and the varying phenotypes of isolates within a single species (Rautelin, et al., 1999), other methods of detecting and identifying *Campylobacter* are attractive alternatives. Molecular assays for *Campylobacter* and other bacteria (Hoorfar, et al., 2000; Sharma, et al., 1999) offer the means to detect the presence of bacteria without culture.

The polymerase chain reaction (PCR) is one of the most widely used molecular techniques in detection and identification of bacteria (Saiki, et al., 1985), and various protocols for detecting and identifying *Campylobacter spp.* have been developed (Table 2-1). PCR employs Taq polymerase to synthesize double stranded DNA specific to the primers used in the reaction. PCR can be carried out in a thermocycler in which the DNA product doubles every cycle, creating a specific product from a small amount of starting

template. Steps in doing PCR involve DNA extraction, amplification and visualization of PCR product.

DNA extraction There are different protocols for DNA extraction such as phenol-chloroform extraction (Ausubel, et al., 1997), and guanidine-thiocyanate extraction (Boom, et al., 1990). Basically, DNA extraction exploit the ability of DNA to bind to organic salts. This complex can be separated from other cell debris after lysis. Then DNA can be purified using an organic solvent.

The sample matrix and DNA extraction process may substantially affect PCR efficiency (Englen and Kelly, 2000; Ludwig and Schleifer, 2000), resulting in a loss of sensitivity when applied to certain types of samples (Chuma, et al., 1997). Different protocols for DNA extraction have successfully extracted *Campylobacter* DNA from fecal specimens (Lawson, et al., 1998; Rasmussen, et al., 1996; Vanniasinkam, et al., 1999) and food samples (O'Sullivan, et al., 2000). A recently developed immuno-magnetic separation procedure has been used to separate *Campylobacter* cells from foods (Yu, et al., 2001), and has yielded satisfactory results (Waller and Ogata, 2000). Mechanical disruption of cells in the presence of DNAzol has proven useful when extracting DNA from heat-resistant *Campylobacter* in environmental samples (Englen and Kelly, 2000).

Amplification Several different primers have been used to achieve identification of *Campylobacter* to the species level (Lawson, et al., 1998). The most commonly used gene for PCR amplification is the 16S rRNA gene. Several other genes have proven useful, such as *flaA* (Rasmussen, et al., 1996), *flaB* (Rasmussen, et al., 1996), *ceuE* (Houng, et al., 2001), 23S rRNA (Eyers, et al., 1993) and *cadF* (Konkel, et al., 1999).

Visualization of PCR products PCR coupled with ELISA utilizing specific probes (Metherell, et al., 1999) or using restriction fragment length polymorphism (RFLP) profiling has been used to identify species of *Campylobacter* after amplification (Fermer and Engvall, 1999; Hurtado and Owen, 1997; Korolik, et al., 2001; Marshall, et al., 1999). PCR coupled with other product visualizing techniques, such as colorimetric assay (Casademont, et al., 2000; O'Sullivan, et al., 2000), has been developed, allowing automated and rapid reading of results. Some colorimetric systems can yield quantitative results (Waller and Ogata, 2000). A fluorogenic PCR has been developed for identifying *C. jejuni* (Wilson et al., 2000), and has been shown to be more specific than standard phenotypic tests using a conventional test kit (Padungtod, et al., 2002).

Advantages of PCR The advantages of using the PCR technique include increased specificity (Lilja and Hanninen, 2001), sensitivity (Vanniasinkam, et al., 1999) and reduction in time required (Denis, et al., 1999). PCR was shown to yield 97.8% specificity when detecting *C. jejuni* from stool samples, when compared to culture technique (Waegel and Nachamkin, 1996). The detectability level (concentration of bacteria ; CFU/ml) of PCR was lower than plate culture, however, the total number of cells require for detection was much less (Lawson, et al., 1998). PCR was shown to yield comparable result to selective agar for detection of thermophilic *Campylobacter spp.* in food samples (Thunberg, et al., 2000). Fluorogenic PCR cuts down diagnostic time to one hour after DNA extraction (Logan, et al., 2001), compared to the six days required by standard culture methods and three days needed for PCR after enrichment (Denis, et al., 1999; Lilja and Hanninen, 2001). Bypassing the enrichment step by using

immunomagnetic separation of cells resulted in shortening the total turnaround time to eight hours (Waller and Ogata, 2000).

Molecular techniques for determining relationship among *Campylobacter spp.* The main objective for genotyping *Campylobacter spp.* in epidemiological studies is to provide evidence that strains related by epidemiological factors (time, place, host) are also genetically related (Tenover, et al., 1995). It was shown over a decade ago that genotyping techniques offer greater discriminatory power than biotyping or phage typing of *Campylobacter* (Patton, et al., 1991). An extensive review of genotyping techniques for *Campylobacter* (Wassenaar and Newell, 2000) included fla-typing (PCR-Randon Amplified Fragment-Length Polymorphism; PCR-RFLP) , pulsed-field gel electrophoresis (PFGE), ribotyping, and random amplified polymorphic DNA (RAPD) as major molecular techniques employed for genotyping of *Campylobacter* (Table 2-2). Other genotyping techniques for *Campylobacter spp.* include Amplified fragment length polymorphism (AFLP), multiplex PCR-RFLP, and nucleotide sequencing (Wassenaar and Newell, 2000).

AFLP is based on complete digestion of whole chromosomal DNA followed by selective amplification of the digested product with specific restriction site recognized by the specific amplification primers (Wassenaar and Newell, 2000). AFLP has been used successfully to identify species, subspecies, and genetic relationships of *Campylobacter spp.* (Duim, et al., 2000; On and Harrington, 2001; Wagenaar, et al., 2001). AFLP is more specific than phenotyping or ribotyping in ability to identify *C. fetus* to species level (Wagenaar, et al., 2001).

PCR-RFLP has been used in epidemiological studies of samples from humans (Waegel and Nachamkin, 1996), animals (Chuma, et al., 1997; Petersen and On, 2000), and is sometimes called macrorestriction profiling (Petersen and On, 2000) or flagellin typing when the *fla* gene is used (Nachamkin, et al., 1993). Because the *fla* gene of *Campylobacter* contains both conserved and variable sequences, primer can be designed to amplify the conserved target areas. Then restriction enzymes can be used to digest the amplified product which will show different banding patterns among different genotype due to variability at the locus (Nachamkin, et al., 1993). Because flagella typing is based on only one genetic locus, it may be able to discriminate at the species level (De Boer, et al., 2000). RFLP was found to correlate well with results from heat-labile serotyping, and offers higher discriminatory power (Nachamkin, et al., 1993).

RAPD is most useful when no previous information on the target bacteria is available. This procedure has been shown to be useful for discriminating virulent strains of *Campylobacter* (Carvalho, et al., 2001), typing rare *Campylobacter* (Misawa, et al., 2000) or characterizing serologically non-typable *Campylobacter* (Mazurier, et al., 1992). RAPD use arbitrary primers to amplify chromosomal DNA under low stringency conditions. Generally, 10-mer primers are used under condition that allow mismatch amplification, which occur randomly throughout the chromosome (Wassenaar and Newell, 2000).

PFGE is one of the most popular techniques and is most widely used in epidemiological studies (Fitzgerald, et al., 2001; Fujita, et al., 1995; Hanninen, et al., 1998) and outbreak investigations (Fitzgerald, et al., 2001; Olsen, et al., 2001). In PFGE, restriction enzyme(s) are used to digest chromosomal DNA in *situ* (immobilize in agarose

prior to cell lysis). After digestion, electrophoresis of the product is achieved under conditions in which the electric field is changed in a pulsed manner (Wassenaar and Newell, 2000). The choice of enzymes using in PFGE varies significantly, but the most discriminatory enzymes found for typing *Campylobacter* so far are *SmaI* combined with *SalI* (Fitzgerald, et al., 2001; Steele, et al., 1998), *HaeIII*, *HindIII*, *PruII*, and *PstI* (Fayos, et al., 1992; Owen, et al., 1993). PFGE was shown to be reproducible in different laboratories (Ribot, et al., 2001), but PFGE profiles may change following several passages of the bacteria on culture media (On, 1998).

Ribotyping is another form of genotyping based on agarose gel electrophoresis of digested genomic DNA, followed by Southern blot hybridization with rRNA gene specific probe (Wassenaar and Newell, 2000). Ribotyping has been shown to be more discriminatory than serotyping in some instances (Smith, et al., 1998), but there was no direct association between ribotypes and serotypes of *Campylobacter* (Fitzgerald, et al., 1996). Species discrimination among *Campylobacter* using automated ribotyping was shown to be unreliable (De Boer, et al., 2000), and the discriminatory power of ribotyping to type *C. jejuni* is limited because *C. jejuni* only have 3 copies of rRNA genes (Wassenaar and Newell, 2000).

When comparing these techniques, AFLP has the highest discriminatory power when typing *C. jejuni* and *C. coli* (De Boer, et al., 2000; Lindstedt, et al., 2000). PFGE (using SalI, SmaI, or both) was more discriminatory than ribotyping, flagellin typing (De Boer, et al., 2000), fatty acid profile typing, serotyping, and biotyping (Steele, et al., 1998). Overall, the ranking of the discriminatory power of these tests, from highest to

lowest, is AFLP, PFGE, PCR-RFLP, and ribotyping (De Boer, et al., 2000; Lindstedt, et al., 2000).

One problem affecting the reproducibility of any genotyping method is the genetic instability of the target DNA, which may occur through recombination, transformation or mutation. Techniques utilizing whole genomic DNA that generate several small bands, such as RAPD, PFGE and AFLP, should be the least sensitive to genomic instability (Wassenaar and Newell, 2000). Additionally, PCR-RFLP should be relatively reproducible, if the RFLP target genes are stable (Wassenaar and Newell, 2000).

Molecular techniques for identifying markers of virulence and resistance in *Campylobacter spp.* Molecular techniques can be used to identify new virulence factors (Carvalho, et al., 2001), and known genetic markers for virulence in *Campylobacter* (Wilson, et al., 2000). Carvalho et al. reported finding of invasive-associated marker in *C. jejuni* and *C. coli* resulting from examination of RAPD profiles (Carvalho, et al., 2001).

There is a large body of work on markers for resistance in *Campylobacter*. A marker has been identified for the efflux mechanism of *C. jejuni*, with is associated with resistance to more than one antimicrobial agent (multi-resistance) (Charvalos, et al., 1995). It has been shown that specific mutations in *gryA* or *parC* gene of *C. jejuni* confer resistance to quinolones (Gibreel, et al., 1998; Wang, et al., 1993). Although various mutations have been reported, the Thr-86-to-Ile (C-T transition) was most frequently associated with high levels of resistance to fluoroquinolones (Ruiz, et al., 1998; Wilson et

al., 2000). Several molecular techniques have been developed to detect this mutation in the *gyrA* gene in *C. jejuni*, including fluorogenic PCR (Wilson, et al., 2000), mismatch PCR amplification (Glaab and Skopek, 1999), and nonradioisotopic single strand conformation polymorphism (Charvalos, et al., 1996). These techniques may be of value in screening *C. jejuni* for antimicrobial resistance without conducting in vitro susceptibility testing.

APPLICATIONS OF MOLECULAR TECHNIQUES IN EPIDEMIOLOGICAL STUDIES OF *CAMPYLOBACTER SPP.*

Outbreak investigation. Molecular techniques can provide laboratory evidence that an outbreak resulted from a common source (Hanninen, et al., 1998; Harrington, et al., 1999), or even pin point the source of the outbreak if isolates from suspected sources are available (Olsen, et al., 2001). Although biotyping and serotyping have proven useful in outbreak investigations (Pearson, et al., 2000), numerical analysis resulting from molecular techniques such as PFGE or AFLP offers higher discriminatory power, and the resulting data can be used to compare isolates from wider geographical areas or from different hosts at different times to yield more information.

Outbreak investigations commonly involve verification of the case and determination of the risk factors associated with increased probability of becoming a case. Establishing a genetic relationship between bacteria isolated from the case and suspected source provides very specific evidence of causation. However, since outbreak investigation is commonly conducted retrospectively, as in a case-control study, the

temporal sequence of events should be ascertained before definitive conclusions can be drawn.

Genetic evidence of relationships among isolates. Molecular techniques can be used to compared isolates from various sources to determine whether there is any relationship among them (Chuma, et al., 1997; Duim, et al., 2000; Fitzgerald, et al., 2001). Clarifying relationships among epidemiologically-related isolates may lead to novel hypotheses about the development and transmission of *Campylobacter* infection, which may increase our understanding of how *Campylobacter* disseminates among various species of animals (Misawa, et al., 2000) and environments (Petersen, et al., 2001), and the transmission dynamics of *Campylobacter* in specific hosts (Harrington, et al., 1999) or across hosts (Duim, et al., 2000).

When conducting epidemiological studies, it is desirable to use highly discriminating assays with the ability to generate data which can be shared among various laboratories. Molecular techniques offer both of these qualities, once improvement and standardization of procedures are established. Disease monitoring and surveillance, or other epidemiological studies of *Campylobacter*, using molecular techniques to provide genetic information will yield more precise information and may lead to more interesting hypotheses on the epidemiology of *Campylobacter*.

Detection and identification of the bacteria, virulence factors, and markers for antimicrobial resistance. Molecular techniques can be used to detect and identify *Campylobacter* with virulence factor(s) as mentioned earlier. The use of genetic markers

is of great value in epidemiological or clinical studies involving difficult to measure outcomes such as low levels of bacteria contamination in a food matrix, and can be useful in monitoring or surveillance of *Campylobacter* with specific qualities, such as resistance to antimicrobial agents. Techniques like fluorogenic PCR may offer specific and rapid assays for monitoring *C. jejuni* with virulence or resistance markers in food products. Improving bacteria separation technique from various matrices, such as immunomagnetic separation will also allow shorter time require for bacteria contamination diagnostic. This would allow food producers to better monitor their products for contamination.

In conclusion, molecular techniques offer valuable assistance to the epidemiological study of epidemiology of *Campylobacter*. The selection of specific techniques to employ may depend on the specific needs and resources of different laboratories. Combinations of different protocols can increase the ability to genetically discriminate *Campylobacter*. However, a combination of protocols may result in longer processing time. Standardization of protocols among various laboratories will allow comparison of result, especially those obtained from numerical analysis of genetic materials, such as PFGE and AFLP. PFGE and AFLP are the most promising techniques to provide useful information for the epidemiological study of *Campylobacter*.

Table 2-1. PCR protocols for the detection and identification of *Campylobacter spp.*

Target gene	Discriminate species	Processing of PCR products*	References
16S rRNA, *hip, asp*	Yes	Agarose gel electrophoresis and southern blot	(Lawson, et al., 1998), (Linton, et al., 1997)
16S rRNA	No	Agarose gel electrophoresis	(Vanniasinkam, et al., 1999)
16S rRNA	Yes [1]	Melting peak analysis of biprobes in real time PCR	(Logan, et al., 2001)
16S rRNA	No [2]	RFLP	(Marshall, et al., 1999)
16S rRNA	Yes	ELISA	(Metherell, et al., 1999)
16S rRNA	Yes	Agarose gel electrophoresis	(Denis, et al., 1999)
16S rRNA	Yes	Avidin capture assay	(Waller and Ogata, 2000)
16S – 23S rRNA spacer region	Yes	Southern blot and colorimetric reverse hybridization	(O'Sullivan, et al., 2000)
16S – 23S rRNA spacer region	Yes [1]	PAGE	(Christensen, et al., 1999)
23S rRNA	Yes	RFLP	(Eyers, et al., 1993), (Fermer and Engvall, 1999), (Steinhauserova, et al., 2001),
23S rRNA	Yes	RFLP	[Korolik, 2001 #573; (Korolik, et al., 1995)
23S rRNA	Yes	RFLP	(Hurtado and Owen, 1997)
*fla*A, *fla*B	No	Internal probe hybridization	(Rasmussen, et al., 1996)
*ceu*E	Yes	Agarose gel electrophoresis	(Houng, et al., 2001)
gyrA	Yes	Fluorogenic PCR	(Padungtod, et al., 2002)
IG02 fragment	Yes	Non-radioactive sandwich hybridization	(Casademont, et al., 2000)

[1] (except *C. jejuni* from *C. coli*)
[2] (can separate *C. jejuni* from *C. coli* with additional primer)
* RFLP – randomly amplified fragmented length polymorphism, PAGE – Polyacrylamide agarose gel electrophoresis, ELISA – enzyme linked immunosorbent assay

Table 2-2 Molecular techniques for genotyping *Campylobacter spp.*

Technique	Decription	Strengths	Limitations
Amplified fragmented length polymorphism (AFLP)	Selective PCR amplification of restriction fragments from restriction enzyme digestion of genomic DNA	• Most discriminatory • Construction of database is possible	• Requires access to an automated DNA sequencer
Polymerase chain reaction – random fragment length polymorphism (PCR-RFLP)	PCR amplification of target gene, followed by restriction enzyme digestion of PCR product.	• Cheap, quick, easy	• Discriminatory power depends on choice of gene and enzyme. • Variation of procedures limit comparison of results
Random amplified polymorphic DNA (RAPD)	Amplification of genomic DNA with arbitrary set of primers using low-stringency polymerase chain reaction	• Does not require prior knowledge of the DNA template • Scans entire genome for differences	• Poor reproducibility • Subjective interpretation of results
Pulsed-field gel electrophoresis (PFGE)	Lysed cells in agarose block followed by digestion of chromosomal DNA with restriction enzyme and electophoreses in pulse changing electric field	• High resolution	• Time and labor intensive • Requires special electrophoresis apparatus • Variation limits comparison of results
Ribotyping	Agarose gel electrophoresis of digested genomic DNA, then hybridized with rRNA gene probe in southern blot.	• Automated system available • High throughput	• Limited discriminatory power • High cost

REFERENCES

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Altekruse, S., D. Swerdlow, and N. Stern. 1998. *Campylobacter jejuni*. Veterinary clinics of North America: Food animal practice. 14(1):31-40.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1997. Current protocols in molecular biology, vol. 1-3. John Wiley & son, New York, NY.

Blaser, M. 1997. Epidemiology and clinical features of *Campylobacter jejuni* infections. The journal of infectious diseases. 176(suppl 2):s103-s105.

Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. W. Dillen, and J. Woordaa. 1990. Rapid and simple method for purification of nucleic acids. Journal of clinical microbiology. 28(3):495-503.

Carvalho, A., G. Ruiz-Palacios, P. Ramos-Cervantes, L. Cervantes, X. Jiang, and L. Pickering. 2001. Molecular characterization of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. Journal of clinical microbiology. 39(4):1353-1359.

Casademont, I., C. Bizet, D. Chevrier, and J. Guesdon. 2000. Rapid detection of *Campylobacter fetus* by polymerase chain reaction combined with non-radioactive hybridization using an oligonucleotide covalently bound to micro wells. Molecular and cellular probes. 14:233-240.

Charvalos, E., E. Peteinaki, I. Spyridaki, S. Manetas, and Y. Tselentis. 1996. Detection of ciprofloxacin resistance mutations in *Campylobacter jejuni gyrA* by nonradioisotopic single strand conformation polymorphism and direct DNA sequencing. Journal of clinical laboratory analysis. 10:129-33.

Charvalos, E., Y. Tselentis, M. Hamzehpour, T. Kohler, and J. Pechere. 1995. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrobial agents and chemotherapy. 39(9):2019-2022.

Christensen, H., K. Jorgensen, and J. E. Olsen. 1999. Differentiation of *Campylobacter coli* and *C.jejuni* by length and DNA sequence of the 16S-23S rRNA internal spacer region. Microbiology. 145:99-105.

Chuma, T., K. Makino, K. Okamoto, and H. Yugi. 1997. Analysis of distribution of *Campylobacter jejuni* and *Campylobacter coli* in broiler by using restriction fragment polymorphism of flaggelin gene. Journal of veterinary medical scienes. 59(11):1011-1015.

Chuma, T., K. Yano, H. Omori, K. Okamoto, and H. Yugi. 1997. Direct detection of *Campylobacter jejuni* in chicken cecal contents by PCR. Journal of veterinary medical science. 59(1):85-87.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of *Campylobacters*. International journal of food microbiology. 26(1):43-76.

De Boer, P., B. Duim, A. Rigter, J. Van Der Plas, W. Jacobs-Reitsman, and J. Wagenaar. 2000. Computer assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. Journal of clinical microbiology. 38(5):1940-1946.

Deming, M., R. Tauxe, P. Blake, S. Dixon, B. Fowler, S. Jones, E. Lockamy, C. Patton, and R. Sikes. 1987. *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. American journal of epidemiology. 126(3):526-534.

Denis, M., C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat, and P. Colin. 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *Campylobacter coli*. Letters in applied microbiology. 29:406-410.

Duim, B., C. Ang, A. Van Belkum, A. Rigter, N. Van Leeuwen, H. Endtz, and J. Wagenaar. 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barre or Miller-Fisher syndrome. Applied and environmental microbiology. 66(9):3917-3923.

Effler, P., M. Ieong, A. Kimura, M. Nakata, R. Burr, E. Cremer, and L. Slutsker. 2001. Sporadic *Campylobacter jejuni* infections in Hawaii: Association with prior antibiotic use and commercially prepared chicken. The journal of infectious diseases. 183:1152-1155.

Englen, M., and L. Kelly. 2000. A rapid DNA isolation procedure for the identification of *Campylobacter jejuni* by polymerase chain reaction. Letters in applied microbiology. 31:421-426.

Eyers, M., S. Chapelle, G. V. Camp, H. Goosens, and R. D. Wachter. 1993. Discrimination among thermophilic *Campylobacter* species by polymerase chain reaction amplification of 23S rRNA gene fragment. Journal of clinical microbiology. 31(12):3340-3343.

Fayos, A., R. Owen, and M. Desai, Hernandez, J. 1992. Ribosomal RNA gene restriction fragment diversity amongst Lior biotypes and penner serotype of *Campylobacter jejuni* and Campylobacter coli. FEMS microbiology letters. 95:87-94.

Fermer, C., and E. O. Engvall. 1999. Specific PCR identification and differentiation of the thermophilic *Campylobacters*, *Campylobacter jejuni*, *C.coli*, *C.lari* and *C. upsaliensis*. Journal of clinical microbiology. 37(10):3370-3373.

Fitzgerald, C., L. Helsel, M. Nicholson, S. Olsen, D. Swerdlow, R. Flahart, J. Sexton, and P. Fields. 2001. Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. Journal of clinical microbiology. 39(7):2386-2390.

Fitzgerald, C., R. Owen, and J. Stanley. 1996. Comprehensive ribotyping scheme for heat stable serotype of *Campylobacter jejuni*. Journal of clinical microbiology. 34(2):265-269.

Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones. 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. Applied and environmental microbiology. 67(4):1429-1436.

Fujita, M., S. Fujimoto, T. Morooka, and K. Amako. 1995. Analysis of strains of *Campylobacter fetus* by pulsed-field gel electrophoresis. Journal of clinical microbiology. 33(6):1676-1678.

Gallardo, F., J. Gascon, J. Ruiz, M. Corachan, T. Anta, and J. Vila. 1998. *Campylobacter jejuni* as a cause of traveler's diarrhoea: clinical features and antimicrobial susceptibility. Journal of travel medicine. 5:23-26.

Gaudio, P., P. Echeverria, C. Hoge, J. Pitarangsri, and P. Goff. 1996. Diarrhea among expatriate residents in Thailand: correlation between reduced *Campylobacter* prevalence and longer duration of stay. Journal of traveller medicine. 3:77-79.

Gibreel, A., E. Sjogren, B. Kaijser, B. Wretlind, and O. Skold. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. Antimicrobial agents and chemotherapy. 42(12):3276-3278.

Glaab, W. E. and T. R. Skopek. 1999. A novel assay for allelic discrimination that combines the fluorogenic 5' polymerase chain reaction (Taqman) and mismatch amplification mutation assay. Fundamental and molecular mechanism of mutagenesis. 430(1): 1-12.

Hanninen, M., S. Pajarre, M. Klossner, and H. Rautelin. 1998. Typing of human Campylobacter jejuni isolates in Finland by pulse-field gel electrophoresis. Journal of clinical microbiology. 36(6):1787-1789.

Harrington, C., F. Thomson-Carter, and P. Carter. 1999. Molecular epidemiological investigation of an outbreak of *Campylobacter jejuni* identifies a dominant clonal line within Scottish serotype HS55 populations. Epidemiology and infections. 122:367-375.

Hoorfar, J., P. Ahrens, and P. Radstrom. 2000. Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. Journal of clinical microbiology. 38(9):3429-3435.

Houng, H., O. Sethabutr, W. Nirdnoy, D. Katz, and L. Pang. 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. Diagnostic microbiology and infectious disease. 40:11-19.

Hurtado, A., and R. J. Owen. 1997. A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. Journal of clinical microbiology. 35(9):2401-2404.

Kalman, M., E. Szollosi, B. Czermann, M. Zimanyi, S. Szekeres, and M. Kalman. 2000. Milkborne *Campylobacter* infection in Hungary. Journal of food protection. 63(10):1426-1429.

Konkel, M. E., S. A. Gray, B. J. Kim, S. G. Garvis, and J. Yoon. 1999. Identification of enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cadF* virulence gene and its product. Journal of clinical microbiology. 37(3):510-517.

Korolik, V., D. Friendship, T. Peduru-Hewa, D. Alfredson, B. Fry, and P. Coloe. 2001. Specific identification, grouping and differentiation of *Campylobacter jejuni* among thermophilic *campylobacters* using multiplex PCR. Epidemiology and infections. 127:1-5.

Korolik, V., L. Moorthy, and P. Coloe. 1995. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* strains by using Restriction endonuclease DNA profiles and DNA fragment polymorphisms. Journal of clinical microbiology. 33(5):1136-1140.

Lawson, A. J., M. S. Shafi, K. Pathak, and J. Stanley. 1998. Detection of *campylobacter* in gastroenteritis: comparison of direct PCR assay of faecal samples with selective culture. Epidemiology and infection. 121:547-553.

Lilja, L., and M. Hanninen. 2001. Evaluation of a commercial automated ELISA and PCR method for rapid detection and identification of *Campylobacter jejuni* and *C.coli* in poultry products. Food microbiology. 18:205-209.

Lindstedt, B., E. Heir, T. Vardund, K. Melby, and G. Kapperud. 2000. Comparative fingerprinting analysis of *Campylobacter jejuni* subsp. *jejuni* strains by amplified fragment length polymorphism genotyping. Journal of clinical microbiology. 38(9):3379-3387.

Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of clinical microbiology. 35(10):2568-2572.

Logan, J., K. Edwards, N. Saunders, and J. Stanley. 2001. Rapid identification of *Campylobacter spp.* by melting peak analysis of biprobess in real time PCR. Journal of clinical microbiology. 36(6):2227-2232.

Ludwig, W., and K. Schleifer. 2000. How quantitative is quantitative PCR with respect to cell counts? Systemic and applied microbiology. 23:556-562.

Marshall, S., P. Melito, D. Woodward, W. Johnson, F. Rodgers, and M. Mulvey. 1999. Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-Restriction Fragment Length Polymorphism analysis of 16S rRNA gene. Journal of clinical mocrobiology. 37(12):4158-4160.

Mazurier, S., S. Van de Geissen, K. Heuvelman, and K. Wernars. 1992. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Letters in applied microbiology. 14:260-262.

Metherell, L., J. Logan, and J. Stanley. 1999. PCR enzyme link immunosorbent assay for detection and identification of *Campylobacter* species: Application to isolates and stool samples. Journal of clinical microbiology. 37(2):433-435.

Misawa, N., S. Shinohara, H. Satoh, H. Itoh, K. Shinohara, K. Shimomura, F. Kondo, and K. Itoh. 2000. Isolation of *Campylobacter* species from zoo animals and polymerase chain reaction-based random amplified polymorphism DNA analysis. Veterinary microbiology. 71:59--68.

Moore, J. E., and P. G. Murphy. 2000. Inhibition of selective media in the isolation of thermophilic *Campylobacter spp.* from foods. British journal of biomedical science. 57:150-151.

Nachamkin, I., K. Bohachick, and C. Patton. 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. Journal of clinical microbiology. 31(6):1531-1536.

Nachamkin, I., J. Engberg, and F. M. Aaestrup. 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. *In* I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Olsen, S., G. Hansen, L. Bartlett, C. Fitzgerald, A. Sonder, R. Manjrekar, T. Riggs, J. Kim, R. Flahart, G. Pezzino, and D. Swerdlow. 2001. An outbreak of *Campylobacter jejuni* infections associated with food handler contamination: The use of pulse-field gel electrophoresis. The journal of infectious diseases. 183:164-167.

On, S. 1998. In vitro genotypic variation of *Campylobacter coli* documented by pulsed-field gel electrophoretic DNA profiling: implications for epidemiological studies. FEMS microbiology letters. 165:341-346.

On, S., and C. Harrington. 2001. Evaluation of numerical analysis of PFGE-DNA profiles for differentiating *Campylobacter fetus* subspecies by comparison with phenotypic, PCR and 16S rDNA sequencing methods. Journal of applied microbiology. 90:285-293.

O'Sullivan, N. A., R. Fallon, C. Carroll, T. Smith, and M. Maher. 2000. Detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in broiler chicken samples using a PCR/DNA probe membrane based colorimetric detection assay. Molecular and cellular probes. 14(1):7-16.

Owen, R., M. Desai, and S. Garcia. 1993. Molecular typing of thermotolerant species of *Campylobacter* with ribosomal RNA gene patterns. Researh in microbiology. 144(709-720).

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Patton, C., I. Wachsmuth, G. Evins, J. Kiehlbauch, B. Plikaytis, N. Troup, L. Tompkins, and H. Lior. 1991. Evaluation of 10 methods to distinguish epidemic associated *Campylobacter* strains. Journal of clinical microbiology. 29(4):680-688.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Petersen, L., E. Nielsen, and S. On. 2001. Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. Veterinary microbiology. 82:141-154.

Petersen, L., and S. On. 2000. Efficacy of flagellin gene typing for epidemiological studies of *Campylobacter jejuni* in poultry estimated by comparison with macrorestriction profiling. Letters in applied microbiology. 31:14-19.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Poocharoen, L., and C. Bruin. 1986. *Campylobacter jejuni* in hospitalized children with diarrhoea in Chiang Mai, Thailand. Southeast Asian journal of tropical medicine and public health. 17(1):53-58.

Rasmussen, H., J. Olsen, K. Jorgensen, and O. Rasmussen. 1996. Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken fecal sample by PCR. Letters in applied microbiology. 23:363-366.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Rautelin, H., J. Jusufovic, and M. J. Hanninen. 1999. Identification of hippurate-negative thermophilic *Campylobacter*. Diagnostic microbiology and infectious diseases. 35(1):9-12.

Ribot, E., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. Barrett. 2001. Rapid pulse field gel electrophoresis protocol for subtyping *Campylobacter jejuni*. Journal of clinical microbiology. 39(5):1889-1894.

Ruiz, J., P. Goni, F. Marco, F. Gallardo, B. Mirelis, T. Jimenez-de-Anta, and J. Vila. 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of gyrA gene mutations in quinolone-resistant clinical isolates. Microbiology and immunology. 42(3):223-226.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and et al. 1985. Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230:1350-3354.

Sharma, V., E. Dean-Nystrom, and T. Casey. 1999. Semi-automated fluorogenic PCR assays (Taqman) for rapid detection of *Escherichia coli* O157:H7 and other shiga toxigenic *E.coli*. Molecular and cellular probes. 13:291-302.

Smith, S., D. Olukoya, A. Fox, and A. Coker. 1998. Ribosomal RNA gene restriction fragment diversity amongst Penner serotype of *Campylobacter jejuni* and *Campylobacter coli*. Z. Naturforsch. 53c(65-68).

Steele, M., B. McNab, L. Fruhner, S. DeGrandis, D. Woodward, and J. A. Odumeru. 1998. Epidemiological typing of *Campylobacter* isolates from meat processing plants by pulse-field gel electrophoresis, fatty acid profile typing, serotyping, and biotyping. Applied and environmental microbiology. 64(7):2346-2349.

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

Tay, S. T., S. D. Puthucheary, S. Devi, and I. Kautner. 1995. Characterisation of *Campylobacters* from Malaysia. Singapore medical journal. 36:282-284.

Tenover, F., R. Arbeit, R. Goering, P. Mickelsen, B. Murray, D. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-filed gel electrophoresis: criteria for bacterial strain typing. Journal of clinical microbiology. 33(9):2233-2239.

Thunberg, R. L., T. T. Tran, and M. O. Walderhaug. 2000. Detection of thermophilic *Campylobacter spp.* in blood-free enriched samples of inoculated foods by the polymerase chain reaction. Journal of food protection. 63(3):299-303.

Vanniasinkam, T., J. A. Lanser, and M. D. Barton. 1999. PCR for the detection of *Campylobacter spp.* in clinical specimen. Letters in applied microbiology. 28(1):52-56.

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Waegel, A., and I. Nachamkin. 1996. Detection and molecular typing of *Campylobacter jejuni* in fecal samples by polymerase chain reaction. Molecular and cellular probes. 10:75-80.

Wagenaar, J., M. Van Bergen, D. Newell, R. Grogono-Thomas, and B. Duim. 2001. Comparative study using amplified fragment length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. Journal of clinical microbiology. 39(6):2283-2286.

Waller, D., and S. Ogata. 2000. Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in food. Applied and environmental microbiology. 66(9):4115-4118.

Wang, Y., W. M. Huang, and D. E. Taylor. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutation. Antimicrobial agents and chemotherapy. 37(3):457-463.

Wassenaar, T., and D. Newell. 2000. Genotyping of *Campylobacter spp.* Applied environmental microbiology. 66(1):1-9.

Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz. 2000. Identification of Ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. Journal of clinical microbiology. 38(11):3971-3978.

Yu, L., J. Uknalis, and S. Tu. 2001. Immunomagnetic separation methods for the isolation of *Campylobacter jejuni* from ground poultry meats. Journal of immunological methods. 256:11-18.

CHAPTER 3

IDENTIFICATION OF *CAMPYLOBACTER JEJUNI* ISOLATES FROM CLOACAL AND CARCASS SWABS OF CHICKENS IN THAILAND BY USE OF A 5' NUCLEASE FLUOROGENIC PCR ASSAY

ABSTRACT

A rapid PCR-based 5' nuclease fluorogenic PCR assay for identifying *Campylobacter jejuni* was applied to *Campylobacter* isolates from chicken cloacal and carcass swabs collected from chicken farms and a slaughterhouse in Thailand. The primers and probe were based on the sequence of the *gyrA* gene in *C. jejuni*. *C. jejuni* isolates were identified using fluorogenic PCR assay of bacterial cells directly from *Campylobacter* selective agar medium. This assay allowed identification of *C. jejuni* within 1 day after colonies appeared on selective media. The fluorogenic PCR assay yielded comparable results to the conventional phenotypic test kit (kappa = 0.76), but required less time. When species identification by the two methods was in conflict, results were confirmed by PCR-RFLP of 23S rRNA genes. In these instances, the fluorogenic PCR assay identified more isolates of *C. jejuni* correctly than the conventional phenotypic test kit (6 of 7 unidentifiable by conventional test kit). The fluorogenic PCR assay offers a rapid and specific method that outperforms the conventional phenotypic test kit in identification of *C. jejuni* from environmental samples.

INTRODUCTION

Campylobacter has been implicated as a major cause of food-borne disease in many countries, including Thailand (Mead, et al., 1999; Varavithya, et al., 1990). In particular, *Campylobacter jejuni* has been cited as the most frequent cause of food-borne disease (Acheson, 2001), and has been associated with Guillain-Barré syndrome (GBS), a neurodegenerative disease in humans (Allos, 2001). *Campylobacter* is frequently isolated from poultry meat products (Atanassova and Ring, 1999; Ono and Yamamoto, 1999; Uyttendaele, et al., 1999), which have been frequently identified as sources of sporadic cases of campylobacteriosis (Pearson, et al., 2000). Contamination of poultry products can be traced back to farms (Berrang, et al., 1999) where chickens are commonly found with *Campylobacter* (Evans and Sayers, 2000). In addition to its presence in meat products, *Campylobacter* has been shown to rapidly develop resistance to clinically-relevant antimicrobial agents such as fluoroquinolones (Jacob-Reitsma, et al., 1994) Since 1970, there has been substantial improvement in isolation and identification techniques for *Campylobacter*. Common techniques employed to isolate *Campylobacter* include culture on selective growth media and filtration methods (Nachamkin, et al., 2000). There are numerous culture media available for the isolation of *Campylobacter* (Corry, et al., 1995), but the antibiotics included as selective agents to control competing flora may also inhibit some strains of *Campylobacter* (Moore and Murphy, 2000). Identification methods for *C. jejuni* include assays such as the hippuricase test, serological tests, lectin agglutination, and cellular fatty acid profiles, each of which has its own advantages and disadvantages (On, 1996). However, these procedures are time-consuming due to the slow growth rate of *Campylobacter* in culture.

Polymerase chain reaction (PCR) techniques for detection and identification of *Campylobacter* have been developed and successfully applied to samples from foods (Thunberg, et al., 2000), fecal samples (Linton, et al., 1997), and poultry products (Waller and Ogata, 2000). PCR-based assays offer a rapid alternative for identifying *Campylobacter* without sacrificing specificity (On, 1996).

A 5' nuclease fluorogenic PCR was recently developed (Holland, et al., 1991)which can be used to rapidly identify specific target genes or discriminate between different alleles of the same target gene (Lee, et al., 1993). Fluorogenic PCR assays use a non-extendable oligonucleotide hybridization probe that contains a fluorescent reporter dye and a quencher dye. During PCR cycling, the probe first specifically hybridizes to the corresponding template, but is digested by the exonuclease activity of Taq DNA polymerase as it moves along the template strand. This cleavage results in an increase of fluorescent emission reporter dye, which can be measured by fluorescence spectrometry. The use of an internal probe carrying a signal-generating system in combination with target-specific primers increases the specificity of the PCR reaction. The level of fluorescence measured at the end of the PCR cycling provides qualitative information on the presence or absence of nucleic acid target (Livak, et al., 1995). A fluorogenic PCR assay using primers and probes specific for the *gyrA* gene was recently developed in our laboratory. This assay successfully identified and enumerated a variety of laboratory and clinical *C. jejuni* isolates (Wilson, et al., 2000). The present study was conducted to evaluate the performance of this fluorogenic PCR assay for identification of *C. jejuni* in samples derived from poultry farms and slaughterhouses and to compare its performance

with a widely-accepted conventional phenotypic test kit for species identification of *Campylobacter spp.*.

MATERIALS AND METHODS

Sample collection and primary isolation. This study was conducted as part of a three year epidemiological study designed to determine the prevalence and antimicrobial susceptibility profiles of *E. coli*, *Salmonella*, and *Campylobacter* in food animals and meat products in Thailand. Samples were collected during the rainy season from May to July, 2000. One hundred and fifty-five 5-6 week old chickens were randomly selected for sample collection from 3 convenience-sampled chicken farms in Thailand. Farms were selected according to their willingness to participate, and possessed chickens at market age (6-7 weeks) at the time of sample collection. Each farm had from 5000 to 8000 chickens, and were within 20 kilometers from the laboratory facilities at Chiang Mai University. One hundred and three chickens from a slaughterhouse were systematically selected for sample collection after defeathering by selecting two birds from every batch of 10 chickens processed on a single day.

Cloacal swab samples were collected by swabbing inside the cloacal area of each chicken with a sterile cotton swab, which was then placed in Stuart's transport medium (RCM supply, Bangkok, Thailand). At the slaughterhouse, samples were collected from the uneviscerated chicken carcasses after killing and defeathering, before putting in the chilling tank. Cloacal swabs were collected along with swab of the area surrounding the cloaca and under the wing of each bird at the slaughterhouse, using sterile cotton swabs that were placed in Stuart's transport medium. Samples were kept on ice during

transportation and refrigerated until processing, within 12 hours after collection. Swabs were streaked directly onto Karmali agar plates (KSA ; Oxoid, Basingstoke, UK), and incubated in plastic bags at 42°C for up to 48 hours under microaerobic conditions (5% O_2, 10% CO_2), using the Anaerocult C system (Merck ; Whitehouse Station, New Jersey). Suspected *Campylobacter* colonies were confirmed by oxidase test, catalase test, and gram stain. Gram-negative spiral rods that were oxidase and catalase positive were identified as *Campylobacter*, frozen and stored in 30% glycerol with Mueller-Hinton broth at -70EC and shipped to Michigan State University on dry ice. Upon arrival, *Campylobacter* isolates were subcultured onto Brucella agar medium (Becton Dickinson ; Franklin Lake, New Jersey) supplemented with 5% defibrinated sheep blood (BASB ; Cleveland Scientific, Bath, Ohio), and on Karmali agar medium. Agar plates were incubated at 37°C under 5% CO_2 for 36 to 48 hours. A single colony was selected from each plate and subcultured on BASB and KSA agar plates for species identification.

Fluorogenic PCR assay. After bacteria were grown on BASB or KSA at 37°C , 5% CO_2, for 36 to 48 hours, cells were harvested and suspended in Mueller-Hinton broth for chromosomal DNA extraction(Wilson, et al., 2000). Primers JL238 and JL239 and the fluorogenic probe TAQ1, were used. The fluorogenic PCR assay PCR reaction mix contained the following: 1X Taqman buffer (PE Applied Biosystem, Branchburg, NJ), 0.2 mM (each) dNTP (0.4 mM dUTP), 0.5 pmol of each primer/mL, 200 nM fluorogenic probe, 0.05 U of Amplitaq Gold polymerase (Perkin-Elmer)/mL, 0.01 U of Amperase UNG (Perkin-Elmer)/mL, 4.5 mM $MgCl_2$, 0.05% gelatin, 0.01% Tween20. A sterile toothpick was used to transfer bacteria from a single colony from BASB or KSA agar medium into the fluorogenic PCR reaction mix. Prior to initial PCR denaturation, all

Fluorogenic PCR reaction mixtures were incubated at 50°C for 2 min in the presence of Amperase UNG to prevent PCR product carryover. Using 50 mL PCR reactions, initial denaturation was conducted at 95EC for 10 min ; the annealing and polymerization steps were combined at 60°C for 1 min and followed by denaturation at 95EC for 30 s. This process was cycled 40 times. Fluorescence emissions were monitored with an ABI Prism 7700 sequence detection system (Perkin-Elmer). The assay was performed on each sample twice. If the Fluorogenic PCR assay results from identical samples were not consistent, or if they were in conflict with conventional test kit results, PCR-RFLP of the 23S rRNA gene was used to confirm the species identification as described below. *C. jejuni* 43429 and *C. coli* 1777208 were used as positive and negative controls respectively.

Conventional test kit. The API CAMPY test strips (Biomerieux, Marcy l'Etoile, France) were utilized as recommended by the manufacturer. Bacterial cells from BASB agar plates were used to inoculated both portions of the strip. The first half of the test strip, which was incubated at 37°C under aerobic conditions for 24 hours, included tests for urease, reduction of nitrate, esterase, hippurase, gamma glutamyl transferase, reduction of chloride to triphenyl tetrazolium, pyrrolidonyl arylamidase, L-arginine arylamidase, L-aspartatearylamidase, and alkaline phosphatase. The second half of the strip, including tests for production of H_2S; glucose, succinate, acetate, propionate, malate, and citrate assimilation; and susceptibility to nalidixic acid, cefazoline and erythromycin was incubated at 37°C under 5% CO_2 for up to 48 hours. The results of each test were read as specified by the manufacturer, and the final identification was

achieved by referring to the analytical profile index (20890) provided by the manufacturer. *C. jejuni* 43429 was used as positive control.

DNA extraction for PCR-RFLP. Bacterial cells scraped from BASB agar plates inoculated 24 hours earlier were pelleted, and DNA was extracted by standard methods (Ausubel, et al., 1997). Briefly, cells were resuspended in TE buffer (10mM Tris-HCl, 1 mM EDTA[pH8.0]) and lysed with 0.5% sodium dodecyl sulphate in the presence of 100 mg of proteinase K/mL. Cellular debris was removed by complexing with hexadecyltrimethyl ammonium bromide followed by phenol-chloroform extraction and Rnase I digestion. DNA was precipitated with isopropanol, redissolved in TE, and its concentration determined using a DU530 spectrophotometer (Beckman Instruments ; Schaumburg, IL).

PCR-RFLP of the 23S rRNA. PCR-RFLP of the 23S rRNA was used to confirm the identity of *C.jejuni* isolates. The protocol described by Fermer and Engvall (Fermer and Engvall, 1999) was used. The primer pair consisting of THERM1 (5' TAT TCC AAT ACC AAC ATT AGT 3') and THERM4 (5' CTT CGC TAA TGC TAA CCC 3') was used to amplify 491 bp of a highly-polymorphic region of the 23S rRNA gene; RFLP banding patterns of this region were previously shown to be specific for all species of thermophilic *Campylobacter* (Fermer and Engvall, 1999). The PCR reaction mix contained 0.25 mM of each primer/mL, 1X PCR buffer, 1.5 mM $MgCl_2$/mL, 0.1 mM each dNTP, 0.05 u/mL Amplitaq (PE Applied Biosystem ; Branchburg, NJ), and 1.6 ng/mL DNA template. The PTC-100 thermocycler (MJ Research ; Watertown, MA) was used as follows. The cycling protocol was preceded by a 12 min incubation at 94°C. Initial denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C

for 1 min. The 50mL PCR sample was cycled 45 times, followed by a final extension at 72°C for 5 min. PCR products were visualized by electrophoresis on a 2% agarose gel. For species identification, PCR products were digested with *Alu*I and *Tsp*5091 in separate reactions, according to the sequence detection system manufacturer's recommendations (Fermer and Engvall, 1999). DNA of known *Campylobacter* species was used as controls, including *C. jejuni* 43429, *C. coli* 1777208, *C. upsaliensis* 43954, *C. lari* 3121, *C. fetus* 27374 ; *E. coli* 25922 was used as a non-*Campylobacter* DNA control. The resulting band patterns were visualized by electrophoresis on 2% agarose gels, ethidium bromide staining, and UV illumination.

Statistical analysis. Statistical analysis was conducted using SAS v. 8 (SAS Institute, Cary, NC). The kappa statistic was calculated to determine the agreement between results obtained via fluorogenic PCR assay and conventional test kit. Kappa statistic determine the agreement between two tests without making an assumption that one is more efficient than another (Rosner, 1995).

RESULTS

From a total of 361 samples collected from chickens from 3 farms and one slaughterhouse, 143 (40%) yielded presumptive *Campylobacter* after primary isolation and biochemical analyses (Table 3-1). The overall prevalence of *Campylobacter* from chicken cloacal swab samples on the 3 farms was found to be 62%, while the *Campylobacter* prevalence of the same sample type at the slaughterhouse was 41%. But the *Campylobacter* prevalence on the finished carcasses was only 3.9%.

Of the 143 isolates, 86 isolates were transported back to the US in dry ice. They were subsequently kept at –80°C in the lab at Michigan State university. Of those 86 isolates transported and frozen, 79 were recovered (92%) from frozen stocks and were analyzed further by the fluorogenic PCR assay. However, due to limited resources, only 59 were subjected to analysis by conventional phenotypic test kit for species identification (Table 3-2). The remaining isolates were not successfully recovered from frozen stocks. In the 59 isolate subset, good agreement was observed between results obtained from the fluorogenic PCR assay and conventional test kit for identification of *C. jejuni* (kappa = 0.76). Of seven isolates with discordant test results, one was identified as *C. jejuni* by conventional test kit, but was negative by the fluorogenic PCR assay. The remaining six isolates were identified as *C. jejuni* by the fluorogenic PCR assay, but were unidentifiable by conventional test kit. Of these six isolates, two discordant isolates were not successfully recovered from frozen stocks, while the remaining four isolates were analyzed further by PCR-RFLP of the 23S rRNA gene (sample identification numbers 408, 411, 488, and 559). The results of PCR-RFLP supported the fluorogenic PCR assay identification of these isolates as *C. jejuni* (Figure 3-1 and Figure 3-2). Additionally, there were seven isolates that gave inconsistent fluorogenic PCR assay results in two separate runs of the test (one positive and one negative result). Of these, five isolates were recovered and subjected to PCR-RFLP (sample identification numbers 408, 481, 502, 526, and 556). PCR-RFLP supported identification of all five of these isolates as *C. jejuni*. Fluorogenic PCR assay results using cells grown on BASB or KSA and tested in parallel also showed significant agreement (kappa = 0.69), suggesting that direct analysis of cells from either medium is effective.

DISCUSSION AND CONCLUSION

The proportion of cloacal swab samples yielding *Campylobacter* isolates from the slaughterhouse (41%) in this study was higher than levels previously observed in America (20%; Jones, et al., 1991) in a similar sample types. However, the prevalence of *Campylobacter* on chicken farms was comparable to what was previously found in China(61%) (Wu, et al., 2000) , but lower than in Britain (90%;Evans and Sayers, 2000). The lower proportion of isolates found in chicken carcasses was possibly due to the incubation temperature. The selective plates were only incubated at 42EC, eliminating the possibility of detecting non-thermophilic *Campylobacter*, which may have contributed to the low levels detected.

In another study, the conventional test kit was shown to yield good agreement (up to 94% agreement at species level) with a conventional biochemical test panel of 11 tests in identifying species of *Campylobacter* (Shih, 2000). However, the time required to obtain these results was 24 hours after the test strip was inoculated, which followed a 24-48 hours initial culture on a blood agar plate to generate an inoculum for the test. The major advantage of the fluorogenic PCR assay was the reduction of time and labor for obtaining results. Colonies from selective media, such as KSA, yielded comparable results with colonies from enrichment media, such as BASB, providing some flexibility in choice of growth media. Additionally, the fluorogenic PCR assay could be performed as soon as colonies appeared on selective media. This assay eliminated the need to extract DNA or visualize results by gel electrophoresis, which reduced the amount of time and labor considerably. Finally, the fluorogenic PCR assay showed a higher success

rate in detecting *C. jejuni* than the conventional phenotypic test kit under our sampling protocol. We are currently developing primers specific to other *Campylobacter* species, such as *C. coli* (now available), that can be used in place of the primer for *C. jejuni* for rapid and specific identification of these other species (unpublished data).

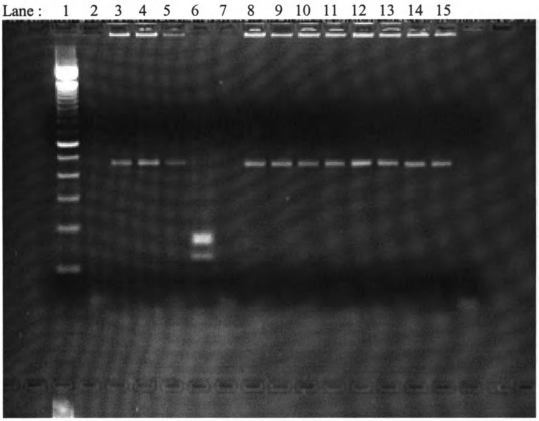
Recently, there have been several studies focused on shortening the detection time for *Campylobacter* in food and fecal specimens. These techniques include PCR of the 16S rRNA (Thunberg, et al., 2000), 23S rRNA (Fermer and Engvall, 1999), and *Fla* (Rasmussen, et al., 1996) genes, multiplex PCR of 16S rRNA, *mapA*, and *ceuE* genes, and enzyme immunoassay (EIA; (Hoorfar, et al., 1999). However, each of these techniques, with the exception of EIA required isolation on selective medium, DNA extraction, and visualization of PCR products by gel electrophoresis with or without Southern blotting. The EIA method, although very rapid, does not allow further susceptibility testing or storage of isolates, since no colonies are obtained. When selective medium is used, it is not possible to precisely estimate the number of *Campylobacter* in the sample, because the selective agent may also inhibit the growth of strains of interest (Moore and Murphy, 2000).

Our fluorogenic PCR assay also yielded reproducible and accurate results when applied directly to identification of *C. jejuni* from selective medium. Although we did not comfirm all the *C.jejuni* positive isolates by our assay with the PCR-RFLP because of limited resources, PCR-RFLP of the discrepant isolates supported the identification by our assay. If the rest of the fluorogenic PCR assay positive isolates were confirmed, it should strengthen the specificity of our assay. Our PCR-RFLP restriction pattern using *Alu*I yielded different patterns for *C.jejuni* from those reported by Fermer and Engvall

(Fermer and Engvall, 1999). This may be the result of different genotypes or the incomplete digestion of the PCR product due to high starting number of templates. We are working on methods to allow us to simultaneously identify and quantify the number of cells in environmental samples without the need to isolate colonies on selective medium. One method under study, immunomagnetic separation (Lea, et al., 1985), has been used successfully in combination with other techniques to detect *Campylobacter* (Lamoureux, et al., 1997) and other food-borne bacteria from various sources (Cudjoe and Krona, 1997; Skjerve, et al., 1990). Immunomagnetic separation was shown to improve the detectability level, particularly in environmental samples(Elder, et al., 2000).

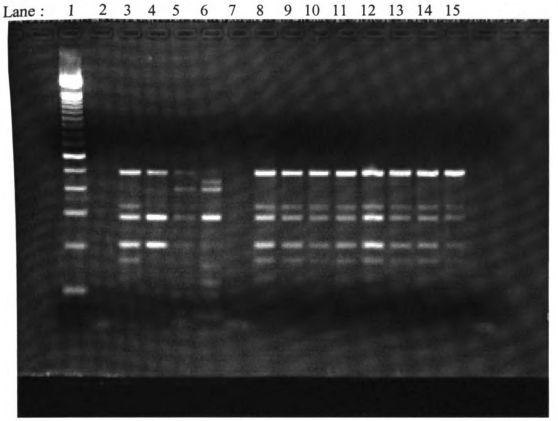
In conclusion, the Fluorogenic PCR assay assay is a sensitive and accurate method for identifying *C. jejuni* directly from a colony on selective medium without the need to extract DNA or perform gel electrophoresis. It can be applied for detection of other species of *Campylobacter*, and is a promising technique, when used in combination with cell separation techniques, to provide a rapid assay for the identification and enumeration of *Campylobacter* in food and environmental samples.

Figure 3-1 PCR-RFLP *Tsp* 5091 digestion patterns.



Lanes: 1) 100 bp DNA ladder; 2) *E. coli* 25922; 3) *C. jejuni* 43429; 4) *C. coli* 1777208; 5) *C. upsaliensis* 43954; 6) *C. lari* 3121; 7) *C. fetus* 27374; 8) 408; 9) 411; 10) 488; 11) 481; 12) 526; 13) 556; 14) 559; 15) 502; 16) no template control

Figure 3-2 PCR-RFLP *Alu* I digestion patterns.



Lanes: 1) 100 bp DNA ladder; 2) *E. coli* 25922; 3) *C. jejuni* 43429; 4) *C. coli* 1777208; 5) *C. upsaliensis* 43954; 6) *C. lari* 3121; 7) *C. fetus* 27374; 8) 408; 9) 411; 10) 488; 11) 481; 12) 526; 13) 556; 14) 559; 15) 502; 16) no template control

Table 3-1 Proportion of samples yielding *Campylobacter* from farms and slaughterhouse

Source	Number of Sample yielding *Campylobacter* / Number of chickens (percent)
Chicken farms	
Farm A	38/55 (69)
Farm B	17/50 (34)
Farm C	42/50 (84)
Total from Chicken farms	97/155 (63)
Slaughterhouses	
Cloacal swab	42/103 (41)
Under wing swab	4/103 (4)
Total from slaughterhouse	46/206 (22)
Grand total	143/361 (40)

Table 3-2 Number of *C. jejuni* samples identified by fluorogenic PCR assay and conventional test kit (n=59).

	Fluorogenic PCR assay	Percent	Conventional test kit	Percent
C.jejuni	33*	56	28*	48
Not *C.jejuni*	26	44	31	52

* **kappa** = 0.76 (95% CI: 0.60 - 0.93) , 1 indicate perfect agreement, 0 indicate no agreement.

REFERENCES

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Atanassova, V., and C. Ring. 1999. Prevalence of *Campylobacter spp.* in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1997. Current protocols in molecular biology, vol. 1-3. John Wiley & son, New York, NY.

Berrang, M. E., R. J. Buhr, and J. A. Cason. 1999. *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. Poultry science. 79:286-290.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of *Campylobacters*. International journal of food microbiology. 26(1):43-76.

Cudjoe, K. S., and R. Krona. 1997. Detection of *Salmonella* from raw food samples using Dynabeads anti-Salmonella and a conventional reference method. International journal of food microbiology. 37(1):55-62.

Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohaemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proceeding of the national academy of sciences. 97(7):2999-3003.

Evans, S. J., and A. R. Sayers. 2000. A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. Preventive veterinary medicine. 46:209-223.

Fermer, C., and E. O. Engvall. 1999. Specific PCR identification and differentiation of the thermophilic *Campylobacters*, *Campylobacter jejuni*, *C.coli, C.lari* and *C. upsaliensis*. Journal of clinical microbiology. 37(10):3370-3373.

Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceeding of the national academy of science USA. 88:7276-7280.

Hoorfar, J., E. M. Nielsen, H. Stryhn, and S. Andersen. 1999. Evaluation of two automated enzyme-immunoassays for detection of thermophilic *campylobacters* in fecal samples from cattle and swine. Journal of microbiological methods. 38:101-106.

Jacob-Reitsma, W. F., C. A. Kan, and N. M. Bolder. 1994. The induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment. Letters in applied microbiology. 19:228-231.

Jones, F., R. Axtell, D. Rives, S. Scheideler, F. Tarver, R. Walker, and M. Wineland. 1991. A survey of *Campylobacter jejuni* Contamination in modern broiler production and processing system. Journal of food protection. 54(4):259-262.

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

Lea, T., F. Vartdal, C. Davies, and J. Ugelstad. 1985. Magnetic monosized polymer particles for fast and specific fractionation of human mononuclear cells. Scandinavian journal of immunology. 22:207-216.

Lee, L. G., C. R. Connel, and W. Bloch. 1993. Allelic discrimination by nick translation PCR with fluorogenic probes. Nucleic acid research. 21:3761-3766.

Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of clinical microbiology. 35(10):2568-2572.

Livak, K. J., S. J. A. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR methods and applications. 4:357-362.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the united states. Emerging infectious diseases. 5(5):607-625.

Moore, J. E., and P. G. Murphy. 2000. Inhibition of selective media in the isolation of thermophilic *Campylobacter spp.* from foods. British journal of biomedical science. 57:150-151.

Nachamkin, I., J. Engberg, and F. M. Aaestrup. 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. *In* I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

On, S. L. W. 1996. Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. Clinical microbiology reviews. 9(3):405-422.

Ono, K., and K. Yamamoto. 1999. Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. International journal of food microbiology. 47:211-219.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Rasmussen, H., J. Olsen, K. Jorgensen, and O. Rasmussen. 1996. Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken fecal sample by PCR. Letters in applied microbiology. 23:363-366.

Rosner, B. 1995. Fundamental of biostatistics, 4 ed. Duxbery press, New York.
Shih, D. Y. 2000. Isolation and identification of enteropathogenic *Campylobacter spp.* from chicken samples in Taipei. Journal of food protection. 63(1):304-308.

Skjerve, E., L. M. Rovik, and O. Olsvik. 1990. Detection of *Listeria monocytogenes* in foods using immunomagnetic separation. Applied and environmental microbiology. 56:3478-3481.

Thunberg, R. L., T. T. Tran, and M. O. Walderhaug. 2000. Detection of thermophilic *Campylobacter spp.* in blood-free enriched samples of inoculated foods by the polymerase chain reaction. Journal of food protection. 63(3):299-303.

Uyttendaele, M., P. D. Troy, and J. Debevere. 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. Journal of food protection. 62(7):735-740.

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Waller, D., and S. Ogata. 2000. Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in food. Applied and environmental microbiology. 66(9):4115-4118.

Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz. 2000. Identification of Ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. Journal of clinical microbiology. 38(11):3971-3978.

Wu, R., G. Liu, and S. She. 2000. Carriage status, isolation and cultivation of *Campylobacter jejuni* from human, domestic animals and poultry. Chinese journal of veterinary science and technology. 30(1):13-16.

CHAPTER 4

PREVALENCE OF *CAMPYLOBACTER SPP.* IN PIGS, FARM WORKERS AND PORK PRODUCTION SYSTEMS IN NORTHERN THAILAND, 2000-2001.

ABSTRACT

A combination of cross-sectional and prospective studies were conducted in pig farms, slaughterhouses and fresh markets in northern Thailand, from 2000-2001 to: 1) compare the frequency of *Campylobacter spp.* on the farm, at the slaughterhouses, and at the market, and 2) identify factors associated with the observed frequency of colonization/contamination.

Fecal samples were collected from 76 pigs at the farm, and the same pigs were followed to the slaughterhouses and markets, where additional samples were collected. Isolation and identification of *Campylobacter* was done using enrichment broth, selective agar, biochemical tests and 5'-nuclease fluorogenic PCR.

The incidence of colonization/contamination was highest at the farms (61.1%), followed by the slaughterhouses (41.7%) and markets (33.3%). Lymph node and fecal samples yielded more *Campylobacter* than carcass swabs from the same pig at the slaughterhouses ($p<0.01$). The prevalence of *Campylobacter* in healthy farm workers was 12.6%. Sampling location and source farms were significantly associated with the observed prevalence.

INTRODUCTION

Campylobacter spp. have been recognized as major food-borne pathogens throughout the world (Acheson, 2001; Pearson, et al., 2000; Phetsouvanh, et al., 1999; Tay, et al., 1995). The majority of human cases of campylobacteriosis are caused by *C. jejuni* and *C. coli* (Nachamkin, et al., 2000). Infection with *C. jejuni* has been reported to result in severe gastroenteritis, which may be fatal in immunocompromised patients (Allos, 2001). One important sequelae of *C. jejuni* infection is Guillain-Barr9 Syndrome (GBS), an acute demyelinating disease of the peripheral nervous system resulting in flaccid paralysis (Acheson, 2001; Altekruse, et al., 1998). Rates of *Campylobacter* infections was 20.1 cases per 100,000 people in the U.S. (Acheson, 2001), 60 – 90 cases per 100,000 people in Europe (Friedman, et al., 2000), and, in Southeast Asia, *Campylobacter* infections were found predominantly in children less than 5 years old, with prevalences ranging from 2.9% - 15% (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988; Taylor, et al., 1993; Varavithya, et al., 1990). Children with *Campylobacter spp.* are often co-infected with *E. coli*, *Salmonella* or *Shigella* (Poocharoen and Bruin, 1986).

Foods of animal origin are commonly implicated as sources of *Campylobacter* infection in humans, both in sporadic cases and disease outbreaks (Acheson, 2001). In developed countries, milk is a common source of *Campylobacter* in disease outbreaks (Evans, et al., 1996; Lehner, et al., 2000), while chicken is a common source of infections for sporadic cases (Effler, et al., 2001; Studahl and Andersson, 2000). Reports of outbreaks of foodborne illness due to *Campylobacter* are rare in developing countries, even though there are reports of *Campylobacter* isolated from foods of animal origin (Rasrinual, et al., 1988).

Campylobacter species most commonly associated with human disease, *C. jejuni* and *C. coli,* have been isolated frequently from domestic animals (Garcia, et al., 1983). In food animal species, pigs commonly carry more *C. coli* than *C. jejuni* (Steinhauserova, et al., 2001). The *Campylobacter* spp. considered to be major veterinary pathogens include *C. fetus*, not commonly found in pigs, and *C. mucosalis* and *C. hyointestinalis*, which have been associated with diseases in pigs (Quinn, et al., 1994).

Because of the increasing public health importance of *Campylobacter spp.* in terms of disease and associated antimicrobial resistance, it is important to study the epidemiology of this infection in both animals and humans. There have been very few studies examining the presence of *Campylobacter* through the food production system from farm to market, in both the food animals and the workers in direct contact with these animals. The basic hypothesis underlying this research is that *Campylobacter spp.* are prevalent in pigs throughout the production system in Thailand, and major factors influencing the observed prevalence can be determined. The objectives of this study, therefore, were to: 1) determine the frequency of *Campylobacter spp.* in pigs and workers at farms, slaughterhouses, and markets, 2) determine the risk factors associated with the observed frequencies.

MATERIALS AND METHODS

Study design and population. This study is a part of a larger epidemiological study of *Campylobacter* in food production systems in northern Thailand. The study was conducted in two phases: the first phase consisted of a cross-sectional study of local pig

producers from May to July of 2000, and the second phase was a prospective study of pigs from local producers from May to July of 2001.

Pig farms under consideration for participation in the study were finishing pig operations, raising pigs from ages 30 to 110 days, that were subcontractors for a company that maintains a large, 1000-sow operation in Chiang Mai province in northern Thailand. In both phases, pig farms were selected to participate in this study based on their willingness to participate. Additional criteria for inclusion were: 1) the farms had to have pigs at the age of approximately 95-100 days (1-2 weeks prior to slaughter) at the first sampling time; and 2) be located within 80 kilometers radius from the laboratory.

Description of the pork production system. Piglets, feed, medication, and veterinary services were provided by the company to each of the contract farms. Once the pigs reached market weight, the company transported the pigs to one of the two municipal slaughterhouses in the Chiang Mai area. All slaughterhouses are government operated and the government provides the facilities and personnel for inspection of animals and carcasses. In general, the company transported the pigs to holding pens at the company headquarters one day before slaughter, and the meat vendors purchased live pigs at the holding pens. The company then delivered the pigs to the specific slaughterhouse selected by the meat vendor, who hired personnel to slaughter and process the carcasses. Once processed, the vendors picked up the carcasses from the slaughterhouse to sell the next morning at the fresh markets.

In the second study phase (year 2001), an arrangement was made with the company and the meat vendors so that the pigs sampled at the farm would be delivered to a specific slaughterhouse and to a specific vendor at the market. This arrangement

allowed us to follow the same pigs from the farm, slaughterhouse, and eventually to the market.

Sample size. Because there were no reports of the prevalence of *Campylobacter* at the farm level in Thailand, the reported prevalence of 12% in food (9) was used to estimate the required sample size. In order to estimate the frequency of *Campylobacter* in pigs within 11% of the true prevalence, and with Type I error of 0.10, a sample size of 22 pigs per farm was derived using a previously published formula (18). To account for attrition, 25 pigs were considered to be an adequate sample size from each farm.

Specimen and data collection. From 10 available pens in pig house at each farm, five pens were systematically selected for the study (every other pen). Each pen had 20-25 pigs, from which five pigs were randomly selected for sample collection. Approximately 10 grams of fecal material were evacuated from the rectum of each pig and stored in plastic cups on ice. Swabs of pen floors and feed trays (pooled sample of two swabs from each pen) were collected using sterile gauze soaked with 10 ml of sterile skim milk. All farm workers were provided with plastic cups containing Cary-Blair medium and asked to submit 10 grams of stool in the cups provided. All samples were kept on ice during transportation and processed within six hours after collection. In the second phase, An ear tag was put on each pig sampled to facilitate tracking of the pig identity from farm through the slaughterhouse and the market.

At slaughter, mesenteric lymph nodes were collected after evisceration. Approximately 20 grams of fecal material were collected from the intestine of each pig. Carcass swabs were collected by wiping an area approximately 40 cm^2 from around the thigh area and inside the rib cage with sterile gauze pads, which were put in plastic bags

with 10 ml sterile skim milk for transport. In the second phase of the study, approximately 100 grams of meat from the neck area attached to the head (with the ear tag) were purchased at the fresh market. Samples were stored on ice during transportation to the laboratory and processed within 12 hours after collection.

Isolation and identification of *Campylobacter spp.* . Fecal samples were directly inoculated, using sterile swabs, on Karmali agar with antimicrobial supplements (including cefoperazone, vancomycin, and amphotericin B), and incubated at 42°C under10% CO_2 for up to 5 days. All swabs were put in 90 ml of Bolton broth with antimicrobial supplements (cefoperazone, trimethoprim, vancomycin, and amphotericin B), and incubated for 48 hours at 42°C under10% CO_2, and a swab of the supernatant from each sample was inoculated on Preston agar with antimicrobial supplements (polymixin B, rifampicin, trimethoprim, and cycloheximide). Approximately 10 grams of the inside of lymph node were collected using sterile equipment, minced, and then processed as described above for swabs.

The agar plates were examined everyday for positive colonies. Suspected *Campylobacter* colonies were confirmed by oxidase test (Dryslide, BBL), catalase test (3% H_2O_2), and gram stain. Gram-negative spiral rods that were oxidase and catalase positive were identified as *Campylobacter*, frozen and stored in 30% glycerol with Mueller-Hinton broth at -70°C for future analysis. *Campylobacter* isolates were subjected to speciation using a 5'-nuclease fluorogenic PCR (Padungtod, et al., 2002).

Statistical analysis. A pig was classified as positive if it had at least one sample with positive *Campylobacter spp.* identification. Incidence was calculated as the number of new positive samples at each sampling point, divided by number of samples with

negative isolation results at previous sampling point. Relative risk was calculated as a ratio of incidence of *Campylobacter* between two sampling locations. Prevalence was calculated as the number of positive samples divided by total number of samples tested. A Chi-square test of independence was used to determine the significance of any association between sampling location and the proportion of positive pigs in 2000.

A multivariable logistic regression model with random effects was used to model the odds of finding *Campylobacter* in pigs at different location in 2001. Pig identification was included in the model as random effect term.

A similar technique was used to model the odds of finding *Campylobacter* in pigs from different farms controling for year of sample collection both at farms and slaughterhouse. Batch of pigs was included in the model as random effect term.

Model parameters were estimated using Generalized Estimating Equation (GEE; Zeger and Liang, 1986). GEE can be applied to repeated measures data with missing value, and consistent estimates can be obtain under mild assumption of correlation among observation (Stokes et al.,2000). All analysis was done using SAS V8.01 (SAS institute Inc., Cary, NC).

RESULTS

A total of five farms, three slaughterhouses, and one fresh market participated in the study. In 2000, three pig farms and Lampoon municipal slaughterhouse participated in the study. In 2001, three pig farms, and the Sansai and Chiang Mai municipal slaughterhouses participated in the study. Two of the three farms participating in 2001 were also participants in 2000. Samples were also collected from Muang Mai market in

Chiang Mai city. A total of 402 samples from pigs, 32 farm worker samples, 92 environmental samples, and 67 pork samples were collected.

Frequencies of *Campylobacter* isolation. *Campylobacter spp.* were isolated at higher rates from the pigs at farms than those at slaughterhouses (Table 4-1). The prevalence of *Camplyobacter* was highest in pigs and lowest in environmental samples. Farm environmental samples yielded higher rate of *Campylobacter* isolation than samples from slaughterhouse environment. The average sample prevalence of *C. jejuni* for both years of study was 1.1%. At slaughterhouses in 2001, significantly more *Campylobacter* were isolated from lymph node samples (68.6%) than carcass swab samples (36.5%; chi-square $p < 0.01$). In 2000, significantly more *Campylobacter* were isolated from fecal samples (21.2%) than carcass swab samples (6.0%; chi-square $p < 0.01$). In 2000, other species of *Camplyobacter* identified included *C. coli* (72.2% of isolates), *C. cryaerophila* (1.3%), *C. fetus* (1.3%), *C. sputorum* (1.3%), and *C. upsaliensis* (1.3%).

The incidence of *Campylobacter* colonization/ contamination was highest at the farms, followed by the slaughterhouse and market respectively (Table 4-2). However, the higher risk of contamination reflected by the relative risk was not statistically significant.

Risk factors. The effect of sampling location on the odds of finding *Campylobacter* in pigs was summarized in Table 4-3. The odds of finding *Campylobacter* in pigs at the farm was significantly higher than slaughterhouse in both years of the study.

The effect of overall management of the farm, which was represented by source farm of the pigs, on the odds of finding *Campylobacter* both at the farm and the slaughterhouse was shown in Table 4-4. Pigs from farm C had significantly higher odds

of being found with *Campylobacter* than other farms. Both at the farm and the slaughterhouse.

DISCUSSION AND CONCLUSION

By prospectively follow the same pigs from farm to slaughterhouse and market, we were able to demonstrate the presence of *Campylobacter* through the pig production system. This study design allowed us to determine the risk of acquiring *Campylobacter* in pigs at farm, slaughterhouse, and market. Other study design such as case-control or cross-sectional study would not be able to clarify the time sequence of events as shown in our study. Our study clearly showed that 61.1% of negative pigs became positive for *Campylobacter* isolation during one week period at the farm, 41.7% of negative pigs at the farms became positive at the slaughterhouse, and 33.3% of negative pigs at the slaughterhouse became positive at the market.

The prevalence of *Campylobacter spp.* observed on the farm was, surprisingly, comparable to what was previously reported in the U.S. (86-99%; Harvey, et al., 1999), and the Netherlands (85%; Weijtens, et al., 1997). The high incidence of colonization observed at the farm may largely be attributed to the open housing in which birds can freely fly through. Wild birds have been implicated as possible source of *Campylobacter* contamination in chicken farms (Jones, et al., 1991). The high prevalence observed may also be the result of colonization before the pigs were transported to the fattening farms. Young (Young, et al., 2000) reported 57.8% colonization in newborn piglets and 100% colonization in weaning pigs in the U.S. Because of the time limitation, we did not sample the pigs before they were initially transported to the farms, which would have

provided information on this possible source of infection. Molecular identification techniques, such as PFGE or PCR-RFLP, could be employed to clarify the relationship among isolates from the same location (Wassenaar and Newell, 2000).

The prevalence of *Campylobacter* in pig house environment samples was lower than from samples collected directly from the pigs. This difference may be due to the sample collection method (Hoar, et al., 1999) or the fact that *Campylobacter* do not survive well in aerobic conditions in the environment. Employing a manure drag through the house may have improved sensitivity of detection in environmental sample (White, et al., 1997).

Our observations confirmed that *C. coli* seems to be the predominant species of *Campylobacter* in pigs in Thailand. Similar observations have been made for pigs in America (Young, et al., 2000) and Europe (Steinhauserova, et al., 2001).

There were significant differences in the prevalence of *Campylobacter* observed in samples from carcass at the slaughterhouse between 2000 and 2001 (chi-square $p < 0.01$). This may be due to differences in the sample collection and isolation protocols between 2000 and 2001. Larger pieces of gauze were used in 2001, which might have increased the number of bacteria picked up when compared to the cotton swabs used in 2000 (Korsak, et al., 1998). Also, the enrichment step of all swab samples was only implemented in 2001, which might have increased the sensitivity of the isolation protocol in the second year (Corry, et al., 1995).

Lymph node and fecal samples yielded more *Campylobacter* than carcass swabs. Unfortunately, lymph node and fecal samples were not collected from the same animal in 2000, therefore comparison between those two type of samples may not be valid.

Although lymph node samples may be more sensitive for detecting *Campylobacter* at the slaughterhouse, the finding may only reflect the level of colonization at the farm, and not the contamination at the slaughterhouse, which carcass swabs may better represent. Fecal samples may reflect colonization at the farm, and may indicate the risk of contamination posed by the carcass at slaughter.

Approximately half of the pigs with no *Campylobacter* at the farms were contaminated at the slaughterhouse. This observation may be the result of increased shedding of the organism following transportation (Whyte, et al., 2001) or contamination from water used in the slaughterhouse (Hanninen, et al., 1998).

The prevalence of *Campylobacter spp.* from meat samples at the market was slightly higher in our study (24.6%) than previously reported in the U.S. (1.3%; Duffy, et al., 2001) and Thailand (12%; Rasrinual, et al., 1988). The reported prevalence of *Campylobacter* in foods from Thailand included other types of meat besides pork (Rasrinual, et al., 1988), which may make comparison of findings inappropriate. The prevalence at the market was comparable to prevalence of *Salmonella* in pork reported in Thailand (24%; Boonmar, et al., 1997).

Four out of thirty-two stool specimens from healthy farm workers yielded *C.jejuni*. This may be the result of asymptomatic *Campylobacter* infection, which is common in adults in developing countries (Allos, 2001). It was also suggested that the isolation of *Campylobacter* from healthy persons in developing countries may be the result of constant reinfection (Oberhelman and Taylor, 2000). This may be the case for farm and slaughterhouse workers, who are frequently in contact with animals with diarrhea (Saeed, et al., 1993).

Sampling location and source farm of the pigs were shown to be associated with the prevalence of *Campylobacter*. Although *Campylobacter* were found at lower level at the market, it may present greater risk to consumer, compared to workers at the farms and slaughterhouse. Prevalence of *Campylobacter* in pig from farm C was much higher than other farms both at the farm and at the slaughterhouse. This observation suggested increase probability of finding *Campylobacter*, in pigs at the slaughterhouse, if the pigs were raised in farm with high prevalence of *Campylobacter*. The fact that workers in farm C also work in the chicken house might have contributed to the higher prevalence observed at farm C, compared to other farms.

In conclusion, our study demonstrated a high prevalence of *Campylobacter spp.* throughout the pig production system in northern Thailand. This poses a direct risk of infection to consumers, particularly children in Thailand. These high prevalence levels, coupled with the documented ability of *Campylobacter* to develop and/or acquire resistance to antimicrobial agents, pose a potentially serious problem of antimicrobial resistance in a widespread human pathogen in Thailand. Long-term epidemiological studies, employing a longitudinal study design, should be conducted to clarify the risk factors for *Camplyobacter* colonization at pig farms. New studies involving more farms should be conducted, as this will allow causal relationship to be established more precisely, with more statistical power.

Table 4-1 Prevalence of *Campylobacter* in pigs and farm workers in northern Thailand, 2000-2001.

Place	Pigs		Workers		Environment	
	# Tested	% Positive	# Tested	% Positive	# Tested	% Positive
Farms	254	73.6	32	12.5	70	14.3
Slaughterhouse	180	45.6	NA	NA	22	4.6
Market	69	24.6	NA	NA	NA	NA

NA – No available sample

Table 4-2 Incidence of *Campylobacter* colonization/contamination in pigs in northern Thailand, 2000-2001.

Place	# Tested	% Positive	Relative risk	
			Risk	95%C.I.
Farms	18	61.1	1.83	0.65-2.54
Slaughterhouse	12	41.7	1.40	0.57-3.43
Market*	15	33.3	-	-

* base for comparison

Table 4-3 Effect of sampling location on the odds of finding *Campylobacter* in pig

Year	Location	Prevalence (%)	Odds ratio	95% CI
2000	Farm	59.6	4.21	2.35 – 7.57
	Slaughterhouse	26.0		
2001	Farm	92.6	4.66	2.30 – 9.45
	Slaughterhouse	72.4	8.28	3.73 – 18.38
	Market	24.6		

Table 4-4 Effect of source farm on the probability of finding *Campylobacter* in pigs at farm and slaughterhouse

Farm	Prevalence at farm (%)	Odds ratio* (95% CI)	Prevalence at slaughterhouse (%)	Odds ratio (95% CI)
A	79.2	2.5 (0.5 – 13.1)	70.6	1.28 (1.0 – 1.7)
B	24.0	0.4 (0.1 – 2.3)	No sample	-
C	92.1	8.9 (1.4 – 55.7)	94.7	9.61 (7.3 – 12.7)
D	88.0	-	62.5	-

*Control for year of sample collection

REFERENCES

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Altekruse, S., D. Swerdlow, and N. Stern. 1998. *Campylobacter jejuni*. Veterinary clinics of North America: Food animal practice. 14(1):31-40.

Boonmar, S., N. Marnrin, S. Pornruangwong, and A. Bangtrakulnonth. 1997. Contamination of *Salmonella* in Chicken and pork products. Kasetsart journal of natural science. 31(4):413-418.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of *Campylobacters*. International journal of food microbiology. 26(1):43-76.

Duffy, E., K. Belk, J. Sofos, G. Bellinger, A. Pape, and G. Smith. 2001. Extent of microbial contamination in United States pork retail products. Journal of food protection. 64(2):172-178.

Effler, P., M. Ieong, A. Kimura, M. Nakata, R. Burr, E. Cremer, and L. Slutsker. 2001. Sporadic *Campylobacter jejuni* infections in Hawaii: Association with prior antibiotic use and commercially prepared chicken. The journal of infectious diseases. 183:1152-1155.

Evans, M., R. Roberts, C. Ribeiro, D. Gardner, and D. Kembrey. 1996. A milk-borne *Campylobacter* outbreak following an educational farm visit. Epidemiology and infections. 117:457-462.

Friedman, C., J. Neimann, H. Wegener, and R. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121-138. *In* I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Garcia, M., M. Eaglesome, and C. Rigby. 1983. *Campylobacter* important in veterinary medicine. Veterinary bulletin. 53(9):793-818.

Hanninen, M., M. Niskanen, and L. Korhonen. 1998. Water as a reservoir for *Campylobacter jejuni* infection in cows studied by serotyping and pulsed-field gel electrophoresis (PFGE). Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 45:37-42.

Harvey, R. B., C. R. Young, R. L. Ziprin, M. E. Hume, K. J. Genovese, R. C. Anderson, R. E. Drolesky, L. H. Stanker, and D. J. Nisbet. 1999. Prevalence of *Campylobacter spp.*

isolated from the intestinal tract of pigs raised in an integrated swine production system. Journal of the american veterinary medical association. 215(11):1601-1604.

Hoar, B. R., E. R. Atwill, C. Elmi, W. W. Uterback, and A. J. Edmondson. 1999. Comparison of fecal samples collected per rectum and off the ground for estimation of environmental contamination attributable to beef cattle. American journal of veterinary research. 60(11):1352-1356.

Jones, F., R. Axtell, D. Rives, S. Scheideler, F. Tarver, R. Walker, and M. Wineland. 1991. A survey of *Campylobacter jejuni* Contamination in modern broiler production and processing system. Journal of food protection. 54(4):259-262.

Korsak, N., G. Daube, Y. Ghafir, A. Chahed, S. Jolly, and H. Vindevogel. 1998. An efficient sampling technique used to detect four foodborne pathogens on pork and beef carcasses in nine Belgian abattoirs. Journal of food protection. 61(5):535-541.

Lehner, A., C. Schneck, G. Feierl, P. Plees, A. Deutz, E. Brandl, and M. Wagner. 2000. Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of *Campylobacter jejuni* in an Austrian youth centre. Epidemiology and infections. 125:13-16.

Nachamkin, I., J. Engberg, and F. M. Aaestrup. 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. *In* I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Oberhelman, R., and D. Taylor. 2000. *Campylobacter* infections in developing countries, p. 139-153. *In* I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Poocharoen, L., and C. Bruin. 1986. *Campylobacter jejuni* in hospitalized children with diarrhoea in Chiang Mai, Thailand. Southeast Asian journal of tropical medicine and public health. 17(1):53-58.

Quinn, P., M. E. Carter, B. A. Markey, and G. R. Carter. 1994. Clinical veterinary microbiology. Wolfe publishing, London.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Saeed, A., N. Harris, and R. DiGiacomo. 1993. The role of exposure to animals in the etiology of *Campylobacter jejuni/coli* enteritis. American journal of epidemiology. 137(1):108-114.

Steinhauserova, I., K. Fojtikova, and J. Matiasovic. 2001. Subtyping of *Campylobacter spp.* strains and their incidence in piglets. Acta veterinaria Brno. 70:197-201.

Stokes, M.E., C.S.Davis, and G.G. Koch. 2000. Categorical data analysis using the SAS system. 2nd ed. SAS Institute, Cary, NC.

Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous *Campylobacter* infection: A swedish case-control study. Epidemiology and infections. 125:269-275.

Tay, S. T., S. D. Puthucheary, S. Devi, and I. Kautner. 1995. Characterisation of *Campylobacters* from Malaysia. Singapore medical journal. 36:282-284.

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

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Wassenaar, T., and D. Newell. 2000. Genotyping of *Campylobacter spp.* Applied environmental microbiology. 66(1):1-9.

Weijtens, M. J. B. M., J. Plas, P. G. H. Bijker, H. A. P. Urling, D. Koster, J. G. Logtestijn, and J. H. J. Veld. 1997. The transmission of *Campylobacter* in piggeries; an epidemiological study. Journal of applied microbiology. 83:693-698.

White, P. L., W. Schlosser, C. E. Benson, C. Maddox, and A. Hogue. 1997. Environmental survey by manual drag sampling for *Salmonella enteritidis* in chicken layer house. Journal of food protection. 60(10):1189-1193.

Whyte, P., J. Collins, K. McGill, C. Monahan, and H. O'Mahony. 2001. The effect of transportation stress on excretion rates of *Campylobacters* in market-age broiler. Poultry sciences. 80:817-820.

Young, C., R. Harvey, R. Anderson, D. Nisbet, and L. Stanker. 2000. Enteric colonization following natural exposure to *Campylobacter* in pigs. Research in veterinary science. 68(1):75-78.

Zeger, S.L., and K.Y.Liang. 1986. Longitudinal data analysis for discrete and continuous outcomes. Biometrics. 42: 121-130.

CHAPTER 5

PREVALENCE OF *CAMPYLOBACTER SPP.* IN BROILER CHICKENS, FARM WORKERS, AND THE CHICKEN PRODUCTION SYSTEM IN NORTHERN THAILAND, 2000-2001

ABSTRACT

A combination of cross-sectional and prospective study design was used to determine the prevalence of *Campylobacter spp.* in broiler chickens at farms, slaughterhouse, market, and human workers associated with those facilities. A total of 6 broiler farms, one slaughterhouse and one meat vendor at the fresh market participated in the study during 2000-2001. A total of 305 fecal swabs from cloaca from the farms, 176 fecal swabs from the cloaca and carcass swabs were collected from the slaughterhouse, 72 thigh muscles were purchased from the market, and 22 stool samples from workers at the farms and slaughterhouse were collected. Isolation and identification of *Campylobacter spp.* was done using enrichment media, selective media and biochemical tests.

Prevalence of *Campylobacter spp.* in broiler chickens was found to be 73.4%, 50.6% and 47.2% at the farms, slaughterhouse and market respectively. *Campylobacter spp.* were significantly more prevalent at the farms ($p \leq 0.01$) than slaughterhouse or market. Significantly more *C. jejuni* were isolated from samples collected at the farms(42.9%; $p \leq 0.01$) than at the slaughterhouse (19.5%) or market (11.8%). No *Campylobacter spp.* were isolated from healthy workers. Older birds was less likely to be colonized with *Campylobacter spp.* ($p \leq 0.01$). Increasing flock size was significantly associated with higher prevalence of *Campylobacter* in chickens, while increasing age was significantly associated with lower prevalence.

INTRODUCTION

The public health consequences of *Campylobacter spp.* are widely recognized. In developed countries, *C. jejuni* has been recognized as a major cause of foodborne bacterial enteritis (Acheson, 2001; Pearson, et al., 2000), and can result in severe gastroenteritis, which may be fatal in immunocompromised patients (Allos, 2001). In contrast to the developed countries, *Campylobacter spp.* has been reported mainly as a diarrheal pathogen in children in developing countries (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988; Taylor, et al., 1993; Varavithya, et al., 1990), and has been reported as prevalent as enterotoxigenic *E. coli* and *Salmonella* in Thailand (Rasrinual, et al., 1988). *Campylobacter* infections in adults in developing countries commonly do not result in any clinical symptom (Allos, 2001).

Foods of animal origin are commonly implicated as sources of *Campylobacter spp.* infection in humans (Acheson, 2001). Chicken is a common source of infection for sporadic cases (Effler, et al., 2001; Studahl and Andersson, 2000) of campylobacteriosis in developed countries. In Thailand, *Campylobacter spp.* was isolated from 12% of food samples (Rasrinual, et al., 1988). *Campylobacter spp.* are commonly isolated from chicken at farms, slaughterhouses and poultry products at retail markets in Germany (Atanassova and Ring, 1999), Denmark (Wedderkopp, et al., 2000), and Japan (Ono and Yamamoto, 1999).

Because of the increasing public health importance of *Campylobacter spp.* in terms of disease and associated antimicrobial resistance, it is important to study the epidemiology of this infection in both animals and humans. There have been very few studies examining the presence of *Campylobacter* through the food production system

from farm to market, in both food animals and the workers in direct contact with these animals. The basic hypothesis underlying this research is that *Campylobacter spp.* are prevalent in chickens throughout the production system in Thailand, and major factors influencing the observed prevalence can be determined. The objectives of our study, therefore, were to: 1) determine the prevalence of *Campylobacter spp.* in broiler chickens at farms, slaughterhouses, and market, and humans working on these farms and slaughterhouses, and 2) determine the risk factors associated with the observed prevalences.

MATERIALS AND METHODS

Study design and population. A combination of cross-sectional and prospective studies were used. This study is part of a larger epidemiological study of *Campylobacter* in food production systems in northern Thailand. The study was conducted in two phases: the first phase consisted of a cross-sectional study from May to July of 2000, and the second phase was a prospective study from May to July of 2001. Chicken farms were selected to participate in this study, based on their willingness to participate. Additional criteria for inclusion were 1) the farms had chickens at the age of approximately 40 days (1 week prior to slaughter) at the first sampling time, and 2) the farms had to be located within 80 kilometers of the laboratory. The chicken farms in the study belonged to two companies, three farms from each company.

Description of the poultry production system. The broiler chicken industry in Thailand is composed of two sectors. The first sector involves large commercial farms that produce meat primarily for export. The second sector involves small farms that

produce meat for the local consumption. Because the latter industry has a major impact on the food safety of the local population, this study was designed to evaluate the problem of *Campylobacter* in the sector that produces meat for local consumption. Each company supplies the farm with newborn chicks, feeds, and medications. The chickens were raised on farms until they reached market age at about 45 to 55 days old. The companies then sell the chickens to meat vendors, who usually operate their own slaughterhouses. After slaughter, the meat vendors sell the chickens at the market the next day. There were no government inspections of animals and carcasses.

In year 2001, an arrangement was made with the company and the meat vendors, so that the chickens sampled from the same flock at the farm would be delivered to a specific slaughterhouse and to a specific vendor at the market. This arrangement allowed us to follow the same flock of chickens from the farm, to the slaughterhouse, and eventually to the market.

Sample size. Because there were no reports of prevalence of *Campylobacter spp.* in chickens at the farm level in Thailand, the reported prevalence of *Campylobacter spp.* in food (12%) was used for sample size calculation (Rasrinual, et al., 1988). In order to estimate the frequency of *Campylobacter* in chickens within 11% of the true prevalence, and with Type I errors of 0.10, a sample size of 22 chickens was derived using a previously published formula (Smith, 1995). To account for any attrition, 25 chickens were considered to be an adequate sample size from each farm.

Specimen and data collection. At the farm, 25 chickens were randomly selected from the chicken house. Fecal swabs were collected from the cloaca, using sterile cotton swabs which were subsequently stored in Stuart's transport media (RCM supply,

Bangkok, Thailand). Samples of floors and water trays (pooled sample of five for each house) were collected using sterile gauze pads soaked with 10 ml of sterile skim milk. All farm workers were provided with plastic cups containing Cary-Blair medium and asked to submit 10 grams of stool in the cups provided. Samples were kept on ice during transportation and processed within six hours after collection. Samples were collected from the farms twice (nine days apart) in 2001. It was not possible to sample the same chickens twice, due to the difficulty in tracking the same chicken from farm to slaughterhouse and market in Thailand.

At the slaughterhouse, samples were collected from the chickens after killing and defeathering but before the carcasses were put into the chilling tank. Fecal swabs from cloaca were collected using sterile cotton swabs and stored in Stuart's transport media. Carcass swabs of the area under both wings were collected using a sterile 25 cm^2 gauze pad, which were subsequently stored in 10 ml skim milk. At the market, a thigh from each chicken was purchased from the vendor. Samples were stored on ice during transportation and processed within 12 hours after collection.

In addition to biological specimens, data concerning farm management and the birds was collected using a pre-tested questionnaire administered to the farm owner by one of the investigators (PP). Information on the birds included the age of birds in the flock being sampled, and the flock size (the number of chickens in the house where samples were collected).

Isolation and identification of *Campylobacter spp.* Fecal samples were directly inoculated on Karmali agar with antimicrobial supplements (including cefoperazone, vancomycin, and amphotericin B), and incubated at 42°C under10% CO_2 for up to 5

days. All other samples were put in 90 ml of Bolton broth with antimicrobial supplements (cefoperazone, trimethoprim, vancomycin, and amphotericin B), and incubated for 48 hours at 42°C under10% CO_2, and a swab of the supernatant from each sample was inoculated on Preston agar with antimicrobial supplements (polymixin B, rifampicin, trimethoprim, and cycloheximide). Approximately 10 grams of meat were removed from each thigh using sterile equipment, minced, and then processed as described above for the non-fecal swab samples.

The agar plates were examined everyday for positive colonies. Suspected *Campylobacter* colonies were confirmed by oxidase test (Dryslide, BBL), catalase test (3% H_2O_2), and gram stain. Gram-negative spiral rods that were oxidase and catalase positive were identified as *Campylobacter*, frozen and stored in 30% glycerol with Mueller-Hinton broth at -70°C for future analysis. *Campylobacter* isolates were subjected to speciation using a 5'-nuclease fluorogenic PCR (Padungtod, et al., 2002).

Statistical analysis. A positive sample was defined as one with positive *Campylobacter spp.* identification. Prevalence was calculated as the number of positive samples divided by total number of samples tested. At slaughter, where two samples were collected from one bird, a positive bird was one where at least one of the samples was positive for *Campylobacter spp.* A Chi-square test was used to determine the significance of the association between proportion of positive birds with the location where samples were taken (farm, slaughterhouse, or market).

A multivariable logistic regression model with random effects was used to model the odds of finding *Campylobacter* in chickens at the farm. Independent variables included: flock size (input in step of 1,000), age of the chickens (day) when samples were

collected, and year of sample collection. A backward elimination algorithm was used. Variable which its removal resulted in change of odds ratio for flock size by 10% was retained in the model. Source farm of the chicken was included in the model as random effect term.

Model parameters were estimated using Generalized Estimating Equation (GEE; Zeger and Liang, 1986). GEE can be applied to repeated measures data with missing value, and consistent estimates can be obtain under mild assumption of correlation among observation (Stokes et al.,2000). All analysis was done using SAS V8.01 (SAS institute Inc., Cary, NC).

RESULTS

In 2000, three chicken farms, with 1000 - 5000 chickens each, from company A participated in the study. In 2001, three chicken farms, with 3000 - 6500 chickens each, from company B participated in the study. The same slaughterhouse participated in both years of the study. Meat samples were collected from Nongdok market in Lampoon city, located 30 kilometers from the laboratory. A total of 553 samples from chickens, 22 from workers, and 37 environmental samples were collected during the study.

Frequency of *Campylobacter* isolation. *Campylobacter spp.* was isolated from the chickens at farms(73.4) more than at slaughterhouses(50.6) or the market (47.2; $p \leq 0.01$; Table 5-1). There was no significant different between the prevalence of *Campylobacter* at the slaughterhouse and market ($p = 0.462$). No *Campylobacter spp.* was isolated from healthy workers, and the prevalence of *Campylobacter* in environmental samples was much lower than in chickens. At the slaughterhouse,

significantly more *Campylobacter* were isolated from cloacal samples (40.8%) than carcass swabs (3.9%; $p \leq 0.01$) in 2000. In 2001, approximately the same proportion of cloacal (34.7%) and carcass(37.5%) swab yielded *Campylobacter* ($p = 0.86$). *Campylobacter* were isolated from carcass swabs significantly more from 2001 samples (37.5%) than from samples taken in 2000 (3.9%; $p \leq 0.01$).

The prevalence of *C. jejuni* isolation was compared with other non-*jejuni* isolates (Table 5-2). The prevalence of *C. jejuni* was higher at the farms than at the slaughterhouse and market ($p \leq 0.01$). There was no significant difference in the prevalence of *C. jejuni* between the slaughterhouse and the market.

Risk factors. The result of a multivariable logistic regression model with random effects was shown in Table 5-3. The final model included only flock size and age of the chickens when samples were collected. Increasing flock size was significantly associated with higher prevalence of *Campylobacter* in pigs at the farm, while increasing age was siignificantly associated with lower prevalence.

DISCUSSION AND CONCLUSION

The prevalence of *Campylobacter spp.* within chicken flocks reported here was similar to reports from other developed and developing countries (Heuer, et al., 2001; Kazwala, et al., 1990; Simango and Rukure, 1991). Only one out of 35 environmental samples from the farms yielded *Campylobacter*. This may reflect the inability of *Campylobacter spp.* to survive in aerobic conditions, where *Campylobacter* may turn into the coccoid non-culturable form (Nachamkin, 1999).

The prevalence of *Campylobacter* at the slaughterhouse was much lower than what was previously reported in modern slaughterhouses(100%) (Berndtson, et al., 1996). The lower observed prevalence in our study probably reflects the differences in slaughter and sample collection processes between modern slaughterhouses and the slaughterhouse in this study. The slaughterhouse in this study was a small local facility that processed only 500-800 chickens per day. Slaughtering was done manually by workers, and carcasses were not opened or eviscerated, which would minimize fecal contamination of the carcasses. Although fecal samples were taken from the cloaca, the fact that the carcasses were not opened may greatly reduce the chance to find *Campylobacter* on those carcasses.

The prevalence of *Campylobacter spp.* observed at the slaughterhouse in 2000 may not be associated with the prevalence observed at the farm since chickens sampled at the slaughterhouse were not from the same flock sampled at the farm. In 2001, although the chickens sampled at the slaughterhouse were from the same flocks sampled at the farm, the *Campylobacter* isolated at the slaughterhouse may not have been those that colonized chickens at the farm. The fact that there was significantly less *C. jejuni* at the slaughterhouse, which was the contrary to what was observed at the farms suggested contamination at the slaughterhouse. The probable source of contamination is water in the slaughterhouse (Berndtson, et al., 1996). Since the carcasses were not opened, there may only be a very limited chance that *C. jejuni* in the chickens would contaminated the carcasses, compared to other modern slaughterhouses (Berrang and Dickens, 2000).

The different prevalences observed from carcass swab samples in 2000 to 2001 may be a result of different sample collection and isolation protocol. A larger piece of

gauze was used to swab the carcasses in 2001, as opposed to the cotton swabs used in 2000. Also, the enrichment step of all swab samples was only implemented in 2001, which might have increased the sensitivity of the isolation protocol in the second year.

The prevalence of *Campylobacter spp.* on meat at the market was higher than previously reported in Thailand (Rasrinual, et al., 1988). However, the previous report was conducted in a different city at different time, and combined various types of meat. Since *C. jejuni* was not very prevalent at the slaughterhouse, it was not surprising that no *C. jejuni* was found on the meat at the market, despite the fact the carcasses were opened and eviscerated at the market before the thigh pieces were purchase from the vendors. This may be the result of evisceration by hand, which may reduce the chance of tearing the intestines in comparison to evisceration using machines.

This study indicated that older chickens were less likely to be colonized by *Campylobacter*, which does not agree with previous studies (Kazwala, et al., 1990). Of flocks sampled in this study, the two flocks with samples collected at age higher than 51 days yielded less *Campylobacter* than other flocks. The previous study which reported the increase in prevalence of *Campylobacter* in chickens only followed birds up to more than 28 days (Kazwala, et al., 1990). This difference in the maximum age of follow up may account for this difference in findings.

Larger flock size was shown to be significantly associated with higher prevalence. This observation may in part be due to the fact that hygiene measures in larger flocks may be less rigorous thanwhat was practiced in smaller flocks. The farms we collected samples from were family owned and operated only by family member. There fore,

limited labour for larger flocksize may play an important role in general hygienic management of the farms.

The reason no *Campylobacter spp.* was found in any farm or slaughterhouse workers was due in part to the fact that there were very few healthy workers available to participate in the study. Five of the six farms participating in the study were operated by family members only, with only two workers per farm. The slaughterhouse was owned and operated by the meat vendor, and employed only four workers. Although it was suggested that the isolation of *Campylobacter* from healthy persons in the developing world was the result of constant reinfection (Oberhelman and Taylor, 2000), we did not observe any *Campylobacter* in workers constantly exposed to *Campylobacter* on the poultry farms or at the slaughterhouse.

In summary, this study demonstrated high prevalences of *Campylobacter* through the chicken production process in northern Thailand. The use of molecular techniques for genotyping *Campylobacter* may be helpful in clarifying the relationships among isolates from different sampling points (Wassenaar and Newell, 2000). This poses a direct risk of infection to consumers, particularly children in developing countries. These high prevalence levels, coupled with the documented ability of *Campylobacter* to develop and/or acquire resistance to antimicrobial agents, pose a potentially serious problem of antimicrobial resistance in a widespread human pathogen in Thailand. Long-term epidemiological studies, employing a longitudinal study design, should be conducted to clarify the risk factors for *Camplyobacter* colonization at pig farms. New studies involving more farms should be conducted, as this will allow causal relationship to be established more precisely, with more statistical power. Studies designed to conduct

analyses at flock level, as opposed to chicken level, will allow more farms to be included in the studies, while maintaining the applicability of results to small poultry producers throughout the area.

Table 5-1. Prevalence of *Campylobacter spp.* in chickens in northern Thailand, 2000-2001.

Place	Chickens		Workers		Environment	
	# Tested	% Positive	# Tested	% Positive	# Tested	% Positive
Farms	305	73.4*	18	0	35	2.9
Slaughterhouse	176	50.6	4	0	2	0
Market	72	47.2	NA	NA	NA	NA

NA – No available sample

* Chi-square $p \leq 0.05$

Table 5-2. Prevalence of *C. jejuni* in chickens in northern Thailand, 2000-2001.

Place	# Tested	% *C. jejuni*	% others	Chi-square p
Farms	205	42.9	57.1	≤0.01
Slaughterhouse	82	19.5	80.5	0.462
Market	34	11.8	88.2	-

– base for comparison

Table 5-3. Results of a multivariable logistic regression model, with random effects, for a single chicken being infected with *Campylobacter* at the farm.

Risk factor	Range	Odds Ratio	95% C.I.
Flock size (*1,000 chickens)	1 - 8	1.3	1.1-1.6
Age of bird (days)	32 - 63	0.90	0.87-0.93

REFERENCE

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Atanassova, V., and C. Ring. 1999. Prevalence of *Campylobacter spp.* in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Berndtson, E., M. Danialsson-Tham, and A. Engvall. 1996. *Campylobacter* incidence on a chicken farm and the spread of Campylobacter during the slaughter process. International journal of food microbiology. 32:35-47.

Berrang, M., and J. Dickens. 2000. Presence and level of *Campylobacter spp.* on broiler carcasses throughout the processing plant. Journal of applied poultry research. 9:43-47.

Effler, P., M. Ieong, A. Kimura, M. Nakata, R. Burr, E. Cremer, and L. Slutsker. 2001. Sporadic *Campylobacter jejuni* infections in Hawaii: Association with prior antibiotic use and commercially prepared chicken. The journal of infectious diseases. 183:1152-1155.

Heuer, O., K. Pedersen, J. Andersen, and M. Madsen. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Letters in applied microbiology. 33:269-274.

Kazwala, R., J. Collins, R. Crinion, and H. O'Mahony. 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commercial poultry production. The veterinary record. 126:305-306.

Nachamkin, I. 1999. *Campylobacter* and *Arcobacter*, p. 716-726. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7 ed. ASM press, Washington DC.

Oberhelman, R., and D. Taylor. 2000. *Campylobacter* infections in developing countries, p. 139-153. *In* I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Ono, K., and K. Yamamoto. 1999. Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. International journal of food microbiology. 47:211-219.

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Simango, C., and G. Rukure. 1991. Potential sources of *Campylobacter* species in the home of farm workers in Zimbabwe. Journal of tropical medicine and hygiene. 94:388-392.

Smith, R. D. 1995. Veterinary Clinical Epidemiology, 2 ed. CRC press, Ann Arbor.

Stokes, M.E., C.S.Davis, and G.G. Koch. 2000. Categorical data analysis using the SAS system. 2nd ed. SAS Institute, Cary, NC.

Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous C*ampylobacter* infection: A swedish case-control study. Epidemiology and infections. 125:269-275.

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

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Wassenaar, T., and D. Newell. 2000. Genotyping of *Campylobacter spp.* Applied environmental microbiology. 66(1):1-9.

Wedderkopp, A., E. Rattenborg, and M. Madsen. 2000. National surveillance of *Campylobacter* in broilers at slaughter in Denmark in 1998. Avian diseases. 44:993-999.

Zeger, S.L., and K.Y.Liang. 1986. Longitudinal data analysis for discrete and continuous outcomes. Biometrics. 42: 121-130.

CHAPTER 6

ANTIMICROBIAL RESISTANCE IN *CAMPYLOBACTER SPP.* ISOLATED FROM FOOD ANIMALS AND HUMANS IN NORTHERN THAILAND, 2000-2001

ABSTRACT

A cross-sectional study was conducted to determine the frequency of antimicrobial resistance in *Campylobacter spp.* isolated from food animals, food products, and farm workers in northern Thailand, and the risk factors associated with the observed frequencies of resistance. Six chicken farms, four pig farms, three slaughterhouses, and two meat vendors participated in the study. Isolation and identification of *Campylobacter* were done using enrichment media, selective media, biochemical tests and fluorogenic PCR. In vitro susceptibility testing was done using the microbroth dilution technique. Antimicrobial agents tested included ciprofloxacin, erythromycin, gentamicin, azithromycin, clindamycin, chloramphenicol, nalidixic acid and tetracycline. Multivariable logistic regression model was used to determine the significance of potential risk factors on the presence of resistance.

Resistance to all 8 antimicrobial agents tested was found in *Campylobacter* isolated from pigs at farms, slaughterhouses and market, and 6 of the agents tested in chickens. The most prevalent forms of resistance seen were to ciprofloxacin, nalidixic acid and tetracycline. The prevalence of resistance was higher for those antimicrobial agents to which animals were exposed. No resistance or low frequency of resistance was observed for those antimicrobial agents not used on the farm. *Campylobacter* isolated from pigs were significantly resistance to more type of antimicrobial agents, and had significantly higher MIC for all antimicrobial agents than those isolated from chickens.

INTRODUCTION

The public health consequences of *Campylobacter spp.* are widely recognized. In developed countries, *C. jejuni* has been recognized as a major cause of foodborne bacterial enteritis (Acheson, 2001; Pearson, et al., 2000), and can result in severe gastroenteritis, which may be fatal in immunocompromised patients (Allos, 2001). In contrast to the developed countries, *Campylobacter spp.* has been reported mainly as a diarrheal pathogen in children in developing countries (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988; Taylor, et al., 1993; Varavithya, et al., 1990), and has been reported as prevalent as enterotoxigenic *E. coli* and *Salmonella* in Thailand (Rasrinual, et al., 1988). *Campylobacter* infections in adults in developing countries commonly do not result in any clinical symptoms (Allos, 2001). Foods of animal origin are commonly implicated as sources of *Campylobacter spp.* infection in humans (Acheson, 2001). *Campylobacter spp.* are commonly isolated from chicken at farms, slaughterhouses and on poultry products at retail markets (Atanassova and Ring, 1999), and in Thailand, *Campylobacter* was isolated from 12% of food samples (Rasrinual, et al., 1988).

Antimicrobial resistance has become one of the major public health concerns in both developed and developing countries in recent years (Isenbarger, et al., 2002; Witte, 1998). Resistance to antimicrobial agents in enteric bacteria poses an increased risk of treatment failure and increased cost of treatment (Vasallo, et al., 1998). The use of antimicrobial agents has been linked to the development of resistant bacteria in humans (Berends, et al., 2001) and livestock (Boonmar, et al., 1998; Jacob-Reitsma, et al., 1994), and levels of antimicrobial resistance appear to be increasing in developing countries (Hoge, et al., 1998), where there is widespread and uncontrolled use of antibiotics (Hart

and Kariuki, 1998). In addition to bacteria showing resistance to individual antimicrobial agents, bacteria have also exhibited resistance to more than one drug (multi-resistance) and cross-resistance between different families of antimicrobials (Isenbarger, et al., 2002; Prescott, 2000).

It is widely speculated that the use of antimicrobial agents in food animal species may be contributing to the antimicrobial resistance problem in humans (Barton, 1998; Hoge, et al., 1998; Witte, 1998). Since *Campylobacter* with resistance to antimicrobial agents has been reported in both developed and developing countries (Isenbarger, et al., 2002; Smith, et al., 1999), the prevalence of potentially antimicrobial-resistant *Campylobacter* in food animals indicates that foods of animal origin may be a source of resistant bacteria for humans (Threlfall, et al., 2000).

In order to determine whether food animals are an important source of bacteria with resistance to antimicrobials, one of the first steps to evaluate this situation is to compare antimicrobial resistance patterns in pathogens from food animals and humans with exposure to these animals. In this study, we hypothesized that *Campylobacter spp.* with resistance to antimicrobial agents are prevalent throughout the food animal production system, and that antimicrobial use in swine and chicken production systems is associated with the frequency of antimicrobial resistance. The objectives of our study, therefore, were to: 1) determine the frequencies of *Campylobacter spp.* with resistance to antimicrobial agents in food animals, food products, and farm workers in northern Thailand, and 2) compare the level and class of antimicrobial resistance in *Campylobacter* isolated from pigs and chickens.

MATERIALS AND METHODS

Study design and sample size. This study is a part of a larger epidemiological study of *Campylobacter* in food production systems in northern Thailand. The study was conducted in two phases: the first phase consisted of a cross-sectional study from May to July of 2000, and the second phase was a prospective study from May to July of 2001.

Because there were no reports of prevalence of *Campylobacter spp.* in chickens and pigs at the farm level in Thailand, the reported prevalence of *Campylobacter spp.* in food (12%) was used for sample size calculation (Rasrinual, et al., 1988). In order to estimate the frequency of *Campylobacter* in food animals with Type I and Type II errors of 0.10, a sample size of 22 animals per farm was derived using a previously published formula (Smith, 1995). To account for any attrition, 25 animals were considered to be an adequate sample size from each farm.

Study population and specimen collection. Six chicken farms, four pig farms, one chicken slaughterhouse, two pig slaughterhouses, and two meat vendors at the market participated in the study. Specimen collection was done during May to July of 2000 and 2001. Pig farms in the study were finishing pig operations, raising pigs from ages 30 to 110 days, that were subcontractors for a company that maintains a large, 1000-sow operation in Chiang Mai province in northern Thailand. The chicken farms in the study belonged to two companies, and raised chickens until they reached market age at about 45 to 55 days old. Farms were selected for this study based on their willingness to participate. Additional criteria for inclusion were: 1) the farms had to have animals of appropriate age at the first sampling time (chickens at the age of approximately 40 days,

pigs at the age of approximately 95-100 days); and 2) be located within 80 kilometers radius from the laboratory.

Specimens were collected at the farm approximately one week before the animals were slaughtered, after slaughter in the slaughterhouse, and at the fresh meat markets (Table 6-1). All samples were stored on ice during transportation to the laboratory and processed within 12 hours after collection.

Animal samples

From available pens in pig house at each farm, five pigs were randomly selected from five systematically sampled pens, and approximately 10 grams of fecal material were evacuated from the rectum of each pig and stored in plastic cups on ice. At the poultry farms, 25 chickens were randomly selected, and fecal swabs were collected from the cloaca, using sterile cotton swabs which were subsequently stored in Stuart's transport media (RCM supply, Bangkok, Thailand).

At slaughter, pig mesenteric lymph nodes were collected after evisceration, approximately 20 grams of fecal material were collected from the intestine of each pig, and carcass swabs were collected by wiping an area approximately 40 cm^2 from around the thigh area and inside the rib cage with sterile gauze pads. Samples were collected from the chickens after killing and defeathering but before the carcasses were put into the chilling tank. Fecal swabs from cloaca were collected using sterile cotton swabs and stored in Stuart's transport media, and carcass swabs of the area under both wings were collected using a sterile 25 cm^2 gauze pad. All carcass swabs were put in plastic bags with 10 ml sterile skim milk for transport.

In the second phase of the study, approximately 100 grams of pork from the neck area attached to the head (with the ear tag) and a thigh from each chicken were purchased at the fresh market.

Farm and slaughterhouse workers samples

Farm and slaugherhouse workers were provided with sterile plastic cups containing Cary-Blair medium and asked to submit 10 grams of stool in the cups provided.

Environmental samples

Swabs of pen floors and feed trays were collected using sterile gauze soaked with 10 ml of sterile skim milk.

Data collection. Data collection was accomplished by using two pre-tested questionnaires that were administered in person by one of the investigators (PP). The first questionnaire was used to collect data relating to the types and quantities of antimicrobial agents used in feed and treatment of sick animals, and other farm management practices. Other data, including the age of the animals sampled, were also collected. The second questionnaire was used to collect data relating to the farm workers. These data included worker's age, gender, medical history, and whether they had consumed any antimicrobial agents within one month prior to the day stool samples were collected.

Isolation and identification of *Campylobacter spp.* Isolation of *Campylobacter spp.* was achieved using enrichment media (Bolton broth) and selective agar (Karmali or Preston agar). Suspected *Campylobacter* colonies were confirmed by oxidase test (Dryslide, BBL), catalase test (3% H_2O_2), and gram stain. Gram-negative spiral rods that were oxidase and catalase positive were identified as *Campylobacter*, frozen and stored

in 30% glycerol with Mueller-Hinton broth at -70°C. The stock bacteria were transported from Thailand to Michigan State University (MSU) where in vitro susceptibility testing was done. Identification of *C. jejuni* was done using 5'-nuclease fluorogenic PCR assay (Padungtod, et al., 2002) conducted at MSU.

In vitro susceptibility testing. In vitro susceptibility testing was done using the microbroth dilution method, following guidelines provided by the National Committee on Clinical Laboratory Standards (NCCLS, 1997). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 37°C under microaerophilic conditions (85%N_2, 5% CO_2, 10% O_2). Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were scraped from the BASB with a sterile cotton swab and suspended in 5 ml H_2O. The turbidity was adjusted to a 0.5 McFarland standard using a standard solution, and one ml of the bacterial suspension was then added to 9 ml of Haemophilus testing medium (HTM). The final concentration of the inoculum was approximately 8 x 10^5 CFU/ml.

Customized SensiTitre plates were purchased pre-made from TREK Diagnostic Systems, Inc. with azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline. These antimicrobials were chosen on the basis of their importance in treating human *Campylobacter* infections and to provide diversity in representation of different antimicrobial classes. Antimicrobial concentrations on the plate ranged from 0.03 to 256 ug/ml, depending on the antimicrobial agent, with a range of seven levels of dilution for each agent. *C. jejuni* ATCC33560 was used as a quality control strain since it was shown to produce

repeatable antimicrobial resistance profiles in microbroth dilution testing. Each plate was inoculated by adding 100 ul of the bacterial suspension to the plate using an autoinoculator. Plates were covered with a gas-permeable seal and incubated at 42EC at 5% CO_2 for 48 hours.

The minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial agent that inhibits growth of the bacteria, was recorded manually. Wells with turbidity or an accumulation of bacteria at the bottom were considered positive for bacterial growth. The breakpoints used to categorize isolates as resistant or not resistant for each antimicrobial agent for *Campylobacter spp.* were those recommended by the National Antimicrobial Resistance Monitoring System (NARMS) (Table 6-2).

Statistical analysis. The prevalence of antimicrobial resistance was calculated as the number of samples yielding *Campylobacter* spp with resistance to a given antimicrobial agent divided by total number of samples tested. A chi-square test was used to compare proportion of *Campylobacter* with resistance to antimicrobial agents from different source.

Because the type and duration of antimicrobial use in pig farms was different from those in chicken farms. All pig farms in our study shared similar protocol for using antimicrobial agents. Similarly all chicken farms in our study also shared similar protocol for using antimicrobial agents. The source animal of *Campylobacter* was used as proxy measure of antimicrobial use when comparing antimicrobial resistance.

A Wilcoxon Rank Sum test was used to compare the number of antimicrobial agent to which *Campylobacter* were resistant between *Campylobacter* isolated from pigs

and chickens. And to compare the minimum inhibitory concentration for each antimicrobial agents between *Campylobacter* isolated from pigs and chickens.

A multivariable logistic regression model with random effects was used to model the odds of being resistance to each antimicrobial agent. Thus, 6 separate multivariable models were conducted for each of the 6 antimicrobial agents tested. Independent variables included source animal (pig, chicken), year of sample collection, location where samples were collected, and whether or not *Campylobacter* isolates was *C.jejuni*. Source animal was the main effect of interest. Specific farms or slaughterhouse where samples were collected was included in the model as random effect term. All variables were included in the model initially. The backward elimination algorithm was used. Variable which its removal resulted in change of odds ratio for source animal by more than 10% was retained in the model.

Model parameters were estimated using Generalized Estimating Equation (GEE; Zeger and Liang, 1986). GEE can be applied to repeated measures data with missing value, and consistent estimates can be obtain under mild assumption of correlation among observation (Stokes et al.,2000). All analysis was done using SAS V8.01 (SAS institute Inc., Cary, NC).

RESULTS

Resistance to antimicrobial agents was found in *Campylobacter* from farms, slaughterhouses, and market, in both pigs and chickens (Table 6-3). In chickens, resistance was found for six of eight antimicrobial agent tested. The most prevalent forms of resistance seen were to ciprofloxacin, nalidixic acid and tetracycline. Over 79% of

Campylobacter isolated from chickens were resistant to more than one agent. In pigs, resistance was found for all eight antimicrobial agents tested. The three most prevalent forms of resistance were ciprofloxacin, nalidixic acid and tetracycline. Over 89% of *Campylobacter* isolated from pigs were resistant to more than one antimicrobial agent. Only two *Campylobacter* isolates from pig farm workers were subjected to in vitro susceptibility testing, and both were resistant to ciprofloxacin, nalidixic acid and tetracycline.

Comparison of proportion of *Campylobacter* spp with resistance to antimicrobial agents from the same pig farms between 2000 and 2001 is shown in Figure 6-1. As seen in Figure 6-1, the proportion of *Campylobacter spp.* resistant to the 8 antimicrobial agents remained very high in the two years. Although there were minor decline in the proportion of resistant *Campylobacter spp.* (Figure 6-1), these declines were not significantly different for all antimicrobial agents except for gentamicin($p<0.01$).

Antimicrobial use on the farm was recorded using questionnaires. Although precise estimates of quantities used were not available, concentrations used in feed or water were obtained, and provide a comparative amount of antimicrobial use. In chicken farms, lincomycin and enrofloxacin were used in feed at 2.2 ppm and 5 ppm, respectively. Chlortetracycline was also used in layer population in the same chicken farms we collected samples from. However, none of the chickens we collected sample were exposed directly to chlortetracycline. On pig farms, amoxycillin, chlortetracycline and lincospectin were used in feed at 200 ppm, 2000 ppm and 1000 ppm, respectively. These antimicrobial agents were used as growth promoters in piglets before they were transported to the finishing farms. Gentamicin, amoxycillin, and enrofloxacin were used

on pig finishing farms for therapeutic only. None of the pigs we collected sample from were treated with these agents.

Comparisons were made between the classes of antimicrobials used on animals and the farms and levels of antimicrobial resistance (Figure 6-2). On both pig and chicken farms, the prevalence of resistance was higher for those antimicrobial agents with related drug exposure to the animals. On both pig and chicken farms, the prevalence of resistance *Campylobacter* was higher for those antimicrobial agents used on the farms in feed and/or therapeutically, whether or not the animals were directly exposed to those agents. While no resistance or extremely low frequency of resistance was observed for those antimicrobial agent not used on the farm.

Campylobacter isolated from pigs were significantly ($p<0.01$) resistance to more type of antimicrobial agents (Figure 6-3) and had significantly higher MIC for all antimicrobial agents (Figure 6-4) than those isolated from chickens. The results of multivariable logistic regression models with random effects (Table 6-4) also showed increase odds of being resistance to ciprofloxacin, erythromycin, azithromycin, clindamycin, nalidixic acid and tetracycline in *Campylobacter* isolated from pigs compared to those isolated from chickens. Models for clindamycin and chlorphenicol were not possible due to lack of resistance in isolates from chickens and low frequency of resistance in pigs.

DISCUSSION AND CONCLUSION

This study found antimicrobial resistance to a variety of agents in *Campylobacter* isolates from food animals, farm workers, and meat samples in Thailand. Our study

demonstrated high prevalences of resistant isolates of *Campylobacter* from pigs and chickens, which are known reservoirs for *Campylobacter* in humans (Pearson, et al., 2000). More importantly, this study demonstrate the association between antimicrobial use on the farms and the prevalence of resistance *Campylobacter* in food animals through the production system at farms, slaughterhouses, and markets. This association was demonstated based on the specificity of the relationship between antimicrobial used on the farms and the prevalence of resistance. Our study demonstrate that resistance to the same class of antimicrobial agents used on the farms were observed, while no resistance or low level of resistance were observed for those antimicrobial agents not used on the farms for both pigs and chickens.

When species of animals were used as proxy measure for antimicrobial used, prevalence of resistance and MIC value for *Campylobacter* isolated from pigs which were exposed to antimicrobial agents for longer period of time was significantly higher than those isolated from chickens. Although we demonstrate the strength of association between antimicrobial used on farms reflected by specie of animals and the odds of *Campylobacter* being resistance to those agents, as shown by the odds ratio in Table 6-4. However, this association reflected by comparing specie of animals should be interpreted with caution. Since almost all *Campylobacter* isolated from chickens were *C. jejuni*, the patterns of resistance seen in chickens may only be reflective of resistance patterns characteristic of *C. jejuni*. Differences in levels of resistance between species may also be due to different exposures to antimicrobial growth promoters, and the period of time that the animals remained on the farms. Chickens remained on the farm for less than 60 days, while pigs were raised for more than 100 days at finishing farms. The longer time period

may allow the accumulation of resistance bacteria from the farm environment, and allow longer exposure time to other animals treated with antimicrobials during the raising period.

Our observation was consistent with the observation previously reported by other researchers (Jacob-Reitsma, et al., 1994; McDermott et al., 2002) demonstrating the associations between exposure to antimicrobial agents and the presence of resistant isolates in chickens and pigs. The chickens in our study were exposed to antimicrobial growth promoters in feeds throughout the study period, and pigs were exposed to antimicrobial growth promoters before they entered this study when they were piglets. Patterns of resistance observed in *Campylobacter* isolates from pigs were probably due to colonization of piglets by resistant bacteria before they were shipped to the finishing farm (Young, et al., 2000). Unfortunately, our study design did not allow clarification of temporal relationship between antimicrobial exposure and incidence of resistance *Campylobacter*. A prospective study following the animals from birth to slaughter will be ideal for clarification of the temporal relationship between exposure to antimicrobial and incidence of resistance development.

The biological plausibility that antimicrobial use in feed exerts selection pressure for those bacteria with resistance to the exposed agents was observed not only in *Campylobacter*, but also in several other bacteria (Bager et al. 1997;Witte et al., 2000). Antimicrobial use in the community and hospital also facilitate the resistance development in several pathogenic bacteria (Neu, 1992). For *Campylobacter*, resistance to fluoroquinolone was observed following the introduction of enrofloxacin in food animals in the Netherland (Endtz et al. 1991) and US (Smith et al. 1999). The plausibility

that bacteria with resistance to antimicrobial agents developed on the farms can be transmitted to human was demonstrated as early as 1987 (Levy, 1987). Therefore, it is plausible that antimicrobial use in pigs and chickens farms in northern Thailand may led to the high prevalence of resistance *Campylobacter* observed in our study.

The overall antimicrobial resistance profiles were generally similar among *Campylobacter* isolated from farms, slaughterhouses, and markets within species. No resistance to gentamicin and chloramphenicol was observed in chickens, regardless of sample type or location of sampling. In pigs, resistance to gentamicin and chloramphenicol were only observed in isolates from the farm and slaughterhouse, but at levels that were lower than for other antimicrobial agents. By class of antimicrobial agent, the prevalence of resistance was comparable in both pigs and chickens. The overall prevalence of resistance in isolates from chickens was to 73% to quinolones (ciprofloxacin) and 81% to nalidixic acid, and 98%-100% in pigs. From farm samples, the prevalence of resistance to macrolides (erythromycin and azithromycin) and clindamycin, a lincosamide which commonly shows cross-resistance with macrolides (Prescott, 2000), ranged from 9.8 to 10.4% in isolates from chickens and 63 to 69% in isolates from pigs. The prevalences of resistance to these classes of agents were comparable at slaughterhouse and market in isolates from both animals.

Multi-drug resistance was found in all types of animal samples from all sampling locations. The overall prevalence of *Campylobacter* with resistance to more than one antimicrobial agents was higher in pig samples than chicken samples. This may be due to co-resistance among antimicrobial agents tested. In *Campylobacter,* resistance to fluoroquinolones in is thought to be chromosomally mediated through the mutation in the

gyrA gene (Gibreel, et al., 1998; Ruiz, et al., 1998; Wang, et al., 1993), and resistance to macrolides is due to mutation in the 23S rRNA gene (Engberg, et al., 2001; Jensen and Aarestrup, 2001). One possible mechanism of co-resistance between quinolones and azithromycin is the efflux pump, which may transport both agents out of bacteria cells (Charvalos, et al., 1995). Since there are no reports of such a mechanism in *Campylobacter*, the mechanism of cross-resistance between these two agents is an area that should be investigated (Isenbarger, et al., 2002).

The *Campylobacter* isolated from healthy farm workers were resistant to the same classes of antimicrobial agents commonly use in both human and animals, including tetracycline and fluoroquinolones. The high prevalence of resistance observed in farm workers may be due to personal use of antimicrobial agents (Berends, et al., 2001). It should be noted, however, that interviews of farm workers showed no recollection of using antimicrobial agents during 30 days period prior to stool collection. It has been suggested that antimicrobial use in food animal production may result in the increasing prevalence of bacteria with resistance to antimicrobial agents in humans (Barton, 1998; Witte, 1998). Although there is evidence that problem of antimicrobial resistance in humans results from personal use of antimicrobial agents (Berends, et al., 2001), the high prevalence of resistant bacteria in food animals may pose an additional risk to the public through consumption of foods of animal origin (Threlfall, et al., 2000). Studying patterns of antimicrobial resistance in farm workers and their animals is an approach that can come closer to addressing the role of antimicrobial use in food animals in the development of antimicrobial resistance in humans. Our study collected samples from commercial farms, slaughterhouses and markets supplying meat for local consumption.

Although there were a limited number of farm workers sampled, the study provides interesting results, upon which future studies can be built.

In summary, our study demonstrated high prevalences of *Campylobacter* with resistance to antimicrobial agents throughout the pig and chicken production system in northern Thailand, and the association between antimicrobial use on the farms and resistance to antimicrobial agents in *Campylobacter*. Further studies to clarify the contribution of antimicrobial use to this problem, and assessment of risk to consumers resulting from resistance bacteria in meat, should be conducted. Although any conclusion regarding the quantitative contributions of antimicrobials used in farm is not possible with this study, a surveillance system of food borne outbreaks and antimicrobial use should be put in place in order to detect changes in antimicrobial resistance profiles in pathogens isolated from food animals and humans, and prevent major public health impact resulting from those resistance food borne bacteria.

Table 6-1 Number and type of samples collected from pig and chicken farms in northern Thailand, 2000-2001.

Year	Sampling location	Number of facilities	Sample type	Number of samples
2000	Chicken farm	3	Cloacal swab	155
			Workers' stool	7
	Chicken slaughterhouse	1	Cloacal swab	100
			Carcass swab	100
	Pig farm	3	Pig fecal	150
			Workers' stool	15
	Pig slaughterhouse	1	Pig fecal	103
			Carcass swab	103
2001	Chicken farm	3	Cloacal swab	150
			Workers' stool	11
	Chicken slaughterhouse		Cloacal swab	73
			Carcass swab	73
			Workers' stool	4
	Chicken meat vendor	1	Thigh muscle	72
	Pig farm	4	Pig fecal	115
			Workers' stool	30
	Pig slaughterhouse	1	Lymph node	70
			Carcass swab	75
	Pork vendor	1	Neck muscle	69

Table 6-2 Minimum Inhibitory Concentration (MIC) dilution ranges and breakpoint values for determination of antimicrobial resistance for *Campylobacter*, based on the National Committee on Clinical Laboratory Standards (NCCLS) recommendations

Antimicrobial agents	Range of Concentrations Tested (μg/ml)	Resistance Breakpoint (μg/ml)
Ciprofloxacin	0.03 – 64	≥ 4
Erythromycin	0.12 – 256	≥ 8
Gentamicin	0.12 – 256	≥ 16
Azithromycin	0.03 – 256	≥ 2
Chloramphenicol	1 – 64	≥ 32
Clindamycin	0.06 – 256	≥ 4
Nalidixic acid	0.12 – 128	≥ 32
Tetracycline	0.25 - 256	≥ 16

Table 6-3 Prevalence (%) of *Campylobacter spp.* with resistance to antimicrobial agents, from chickens, pigs, and farm and slaughterhouse workers in northern Thailand, 2000-2001

	Chickens			Pigs			Worker
	Farm	Slaughter	Market	Farm	Slaughter	Market	Farm
N	183	78	32	202	101	14	2
Ciprofloxacin	73.8	92.3	90.6	98.0	88.1	100.0	100.0
Erythromycin	9.8	2.6	3.1	85.6	66.3	50.0	0.0
Gentamicin	0.0	0.0	0.0	18.3	16.8	0.0	0.0
Azithromycin	10.4	2.6	3.1	85.1	63.4	50.0	0.0
Chloramphenicol	0.0	0.0	0.0	2.0	2.0	0.0	0.0
Clindamycin	9.8	2.6	3.1	85.1	69.3	57.1	0.0
Nalidixic Acid	73.2	89.7	87.5	98.0	90.1	100.0	100.0
Tetracycline	72.7	39.7	81.3	98.5	93.1	92.9	100.0
Multi-drug Resistance	79.2	97.4	96.9	95.5	89.1	100.0	100.0

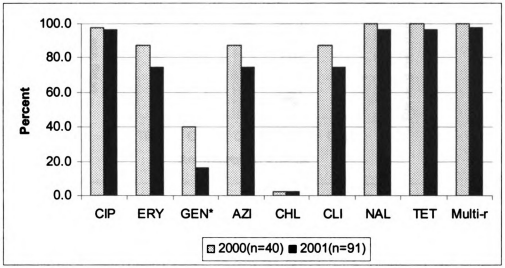
Table 6-4 Odds ratio for resistance in *Campylobacter* isolated from pigs compared to chickens*

Outcome: Resistance to	Odds ratio for pigs**	95% CI
Ciprofloxacin	7.34	2.23 – 24.13
Nalidixic acid	7.18	2.59 – 19.88
Erythromycin	60.36	15.63 – 233.60
Azithromycin	55.94	15.04 – 208.02
Clindamycin	65.15	17.68 – 240.07
Tetracycline	26.75	10.12 – 70.70

*Summary of results of 6 multivariable logistic regression models.

**Adjusted for year of sample collection, location where samples were collected, and whether or not *Campylobacter* isolates was *C.jejuni*.

Figure 6-1 Proportion(%) of *Campylobacter spp.* with resistance to antimicrobial agents from pigs.

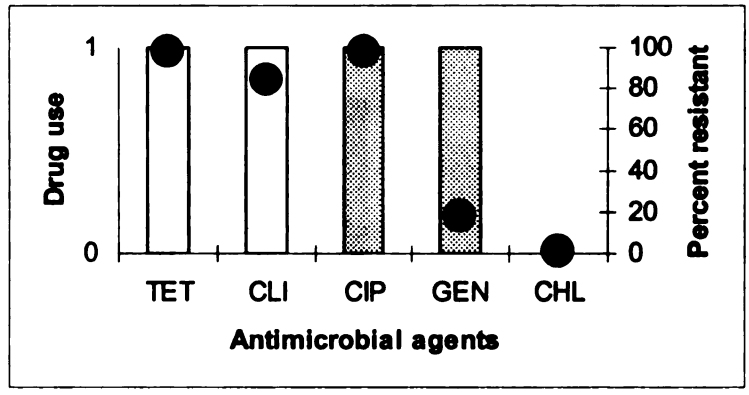


*Significantly different at p≤0.05

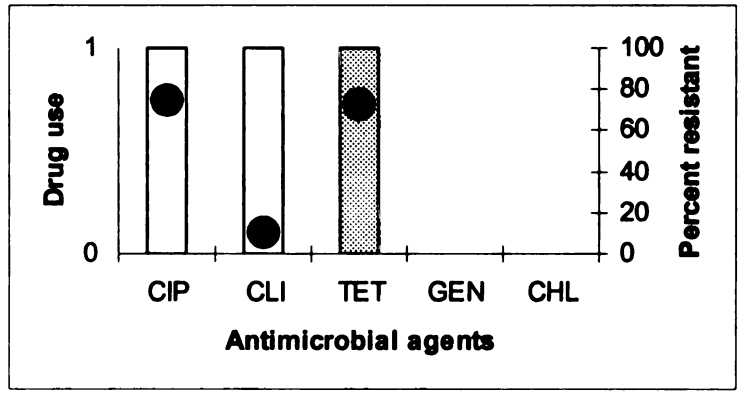
CIP-ciprofloxacin, ERY-erythromycin, GEN-gentamicin, AZI-azithromycin, CHL-chloramphenicol, CLI-clindamycin, NAL-nalidixic acid, TET-tetracycline Multi-r – resistance to more than one agents

Figure 6-2 Relationship between resistance level and antimicrobial use on farms.

A. Pig farms



B. Chicken farms



CIP-ciprofloxacin, CLI-clindamycin, TET-tetracycline, GEN-gentamicin,

CHL-chloramphenicol

□ Antimicrobial use in feed (1 = use, 0 = not use)

▨ Antimicrobial use for treatment / in other group of animal (1 = use, 0 = not use)

● Frequency of resistance

Figure 6-3 Number of antimicrobial agents *Campylobacter spp.* were resistant to.

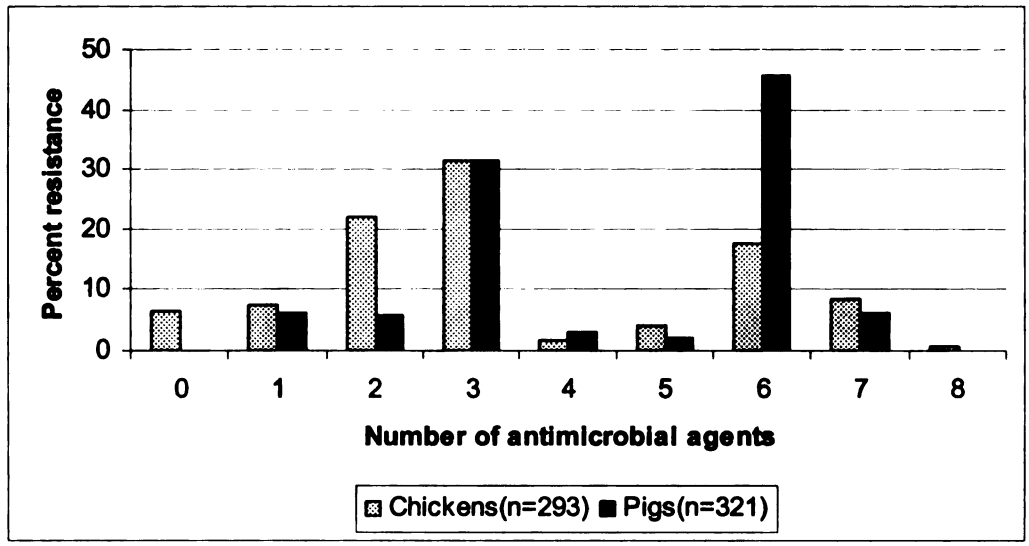
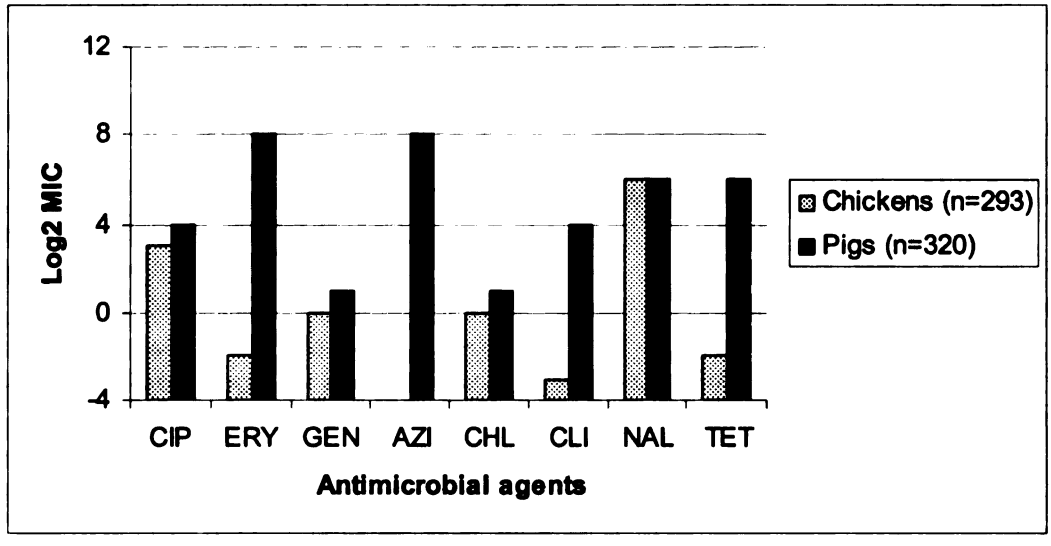


Figure 6-4 Minimum inhibitory concentration of *Campylobacter* isolated from pigs and chickens (mode)



CIP – ciprofloxacin, ERY – erythromycin, GEN – gentamycin, AZI – azithromycin

CHL – chloramphenicol, CLI – clindamycin, NAL – nalidixic acid,

TET - tetracycline

Reference

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Atanassova, V., and C. Ring. 1999. Prevalence of *Campylobacter spp.* in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Barton, M. 1998. Does the use of antibiotics in animals affect human health? Australian veterinary journal. 76(3):177-180.

Bager, F., M. Madsen, J. Christensen, F.M. Aarestrup. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Preventive veterinary medicine. 31:95-112.

Berends, B., A. Van Den Bogaard, F. Van Knapen, and J. Snijders. 2001. Human health hazards associated with the administration of antimicrobials to slaughter animals: Part 2 An assessment of the risks of resistance bacteria in pigs and porks. Vetrinary quarterly. 23:10-21.

Boonmar, S., A. Bangtrakulnonth, S. Pornruangwong, S. Samosornsuk, K. Kaneko, and M. Ogawa. 1998. Significant increase in antibiotic resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. Veterinary microbiology. 62:73-80.

Charvalos, E., Y. Tselentis, M. Hamzehpour, T. Kohler, and J. Pechere. 1995. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrobial agents and chemotherapy. 39(9):2019-2022.

Engberg, J., F. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C.coli*: resistance mechanisms and trends in human isolates. Emerging infectious diseases. 7(1):24-34.

Endtz, H. P., G. J. Rujis, B. Klingeren, W. H. Jansen, T. Reyden, and R. P. Mouton. 1991. Quinolone resistance in *campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. Journal of antimicrobial chemotherapy. 27:199-208.

Gibreel, A., E. Sjogren, B. Kaijser, B. Wretlind, and O. Skold. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. Antimicrobial agents and chemotherapy. 42(12):3276-3278.

Hart, C., and S. Kariuki. 1998. Antimicrobial resistance in developing countries. British medical journal. 317:647-650.

Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsri, and P. Echeverria. 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clinical infectious diseases. 26:341-345.

Isenbarger, D., C. Hoge, A. Srijan, C. Pitarangsi, N. Vithayasai, L. Bodhidatta, K. Hickey, and P. Cam. 2002. Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996-1999. Emerging infectious diseases. 8(2):175-180.

Jacob-Reitsma, W. F., C. A. Kan, and N. M. Bolder. 1994. The induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment. Letters in applied microbiology. 19:228-231.

Jensen, B., and F. Aarestrup. 2001. Macrolide resistance in *Campylobacter coli* of animal origin in Denmark. Antimicrobial agents and chemotherapy. 45(1):371-372.

Lee, C. Y., C. L. Tai, S. C. Lin, and Y. T. Chen. 1994. Occurence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. International journal of food microbiology. 24:161-170.

Levy, S.B. 1987. Antibiotic use for growth promotion in animals: Ecologic and public health consequences. Journal of food protection. 50(7): 616-620.

McDermott, P., S. Bodeis, L. English, D. White, R. Walker, S. Zhao, S. Simjee, and D. Wagner. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. The journal of infectious diseases. 185:837-840.

NCCLS. 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (M37-A4), 4 ed. NCCLS, Wayne, PA.

Neu, H.C. 1992. The crisis in antibiotic resistance. Science. 257:1064-1073.

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Prescott, J. 2000. Lincosamides, macrolides, and pleuromutilins. *In* J. Prescott, J. Baggot, and R. Walker (ed.), Antimicrobial therapy in veterinary medicine, 3 ed. Iowa state university press, Ames.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Ruiz, J., P. Goni, F. Marco, F. Gallardo, B. Mirelis, T. Jimenez-de-Anta, and J. Vila. 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates. Microbiology and immunology. 42(3):223-226.

Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, and M. T. Osterholm. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. The new england journal of medicine. 340(20):1525-32.

Smith, R. D. 1995. Veterinary Clinical Epidemiology, 2 ed. CRC press, Ann Arbor.
Taylor, D. N., D. M. Perlman, P. D. Echeverria, U. Lexomboon, and M. J. Blaser. 1993. *Campylobacter* immunity and quantitative excretion rates in Thai children. The journal of infectious diseases. 168:754-758.

Stokes, M.E., C.S.Davis, and G.G. Koch. 2000. Categorical data analysis using the SAS system. 2nd ed. SAS Institute, Cary, NC.

Threlfall, E., L. Ward, J. Frost, and G. Willshaw. 2000. The emergence and spread of antibiotic resistance in foodborne bacteria. International journal of food microbiology. 62:1-5.

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Vasallo, F. J., P. M. Rabadan, L. Alcala, J. M. G. Lechuz, M. R. Creixems, and E. Bouza. 1998. Failure of ciprofloxacin therapy for invasive nontyphoidal Salmonellosis. Clinical infectious diseases. 26:535-536.

Wang, Y., W. M. Huang, and D. E. Taylor. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutation. Antimicrobial agents and chemotherapy. 37(3):457-463.

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. Science. 279:996-7.

Witte, W., H.Tschape, I. Klare and G. Werner. 2000. Antibiotic in animal feed. Acta veterinary scandinavica. 93 (supplement): 37-45.

Young, C., R. Harvey, R. Anderson, D. Nisbet, and L. Stanker. 2000. Enteric colonisation following natural exposure to *Campylobacter* in pigs. Research in veterinary science. 68(1):75-78.

Zeger, S.L., and K.Y.Liang. 1986. Longitudinal data analysis for discrete and continuous outcomes. Biometrics. 42: 121-130.

CHAPTER 7

USING A FLUOROGENIC PCR ASSAY FOR DETERMINATION OF FLUOROQUINOLONE RESISTANCE IN *CAMPYLOBACTER JEJUNI*

ABSTRACT

A fluorogenic PCR assay for the *gyrA* gene was used to determine the frequency of a Thr-86 mutation in *Campylobacter jejuni* isolates from food animals and humans in northern Thailand, and to investigate the correlation between this mutation and bacterial resistance to fluoroquinolones. A total of 84 isolates of *C. jejuni* were used: 65 from samples from healthy chickens on farms, 16 from chicken samples at slaughterhouse, one from chicken meat at the market, and one isolate from a healthy farm worker. In vitro susceptibility testing was done using the microbroth dilution technique. Minimum inhibitory concentration (MIC) breakpoints established by the National Antimicrobial Resistance Monitoring System were used to categorize resistance in *C. jejuni* to ciprofloxacin and nalidixic acid.

Sixty of the 84 *C. jejuni* isolates tested carried the Thr-86 mutation in the *gyrA* gene. All isolates with ciprofloxacin MIC ≥ 2 :g/ml carried the mutation, and all isolates with nalidixic acid MIC ≤ 16 :g/ml did not carry Thr-86-to-Ile mutation. There was very high agreement between ciprofloxacin resistance and the presence of the mutation (kappa = 0.971, $p \leq 0.01$). The level of agreement between was lower for the presence of the Thr-86-to-Ile mutation with nalidixic acid resistance (kappa = 0.859; $p \leq .01$).

INTRODUCTION

Antimicrobial resistance has become one of the major public health concerns in both developed and developing countries in recent years (Isenbarger, et al., 2002; Witte, 1998). The use of antimicrobial agents has been linked to the development of resistant bacteria in humans (Berends, et al., 2001) and livestock (Jacob-Reitsma, et al., 1994; McDermott, et al., 2002), and levels of antimicrobial resistance appear to be increasing in developing countries (Hoge, et al., 1998), where there is widespread and uncontrolled use of antibiotics (Hart and Kariuki, 1998). Fluoroquinolones have been used successfully for the treatment of severe gastroenteritis in human. The rapid development of fluoroquinolone resistance, particularly in *Campylobacter spp.*, threaten the future usefulness of this class of agents in the future.

Campylobacter spp. has been reported mainly as a diarrheal pathogen in children in developing countries (Phetsouvanh, et al., 1999; Rasrinual, et al., 1988), and infections in adults commonly do not result in clinical symptoms (Allos, 2001). In Thailand, *Campylobacter* has been isolated from 12% of food samples (Rasrinual, et al., 1988), and resistance to tetracycline (Lee, et al., 1994), quinolones and macrolides (Hoge, et al., 1998) has been documented. *Campylobacter spp.* are commonly isolated from chickens at farms, slaughterhouses and on poultry products at retail markets (Atanassova and Ring, 1999), and it has been shown that *Campylobacter* in poultry can rapidly develop resistance following exposure to antimicrobial agents, especially fluoroquinolones (McDermott, et al., 2002).

Fluoroquinolones are bactericidal agents acting by inhibiting the function of DNA gyrase and DNA topoisomerase IV enzymes in bacteria, which leads to a lethal break of

double-strand DNA (Drlica and Zhao, 1997). Resistance to fluoroquinolones in *C. jejuni* was found to be the result of mutation in *gyrA* or *parC*, which encode DNA gyrase and topoisomerase IV, respectively (Gibreel, et al., 1998; Ruiz, et al., 1998; Wang, et al., 1993). A single mutation in *gyrA* gene from Thr-86 to Ile (C to T transition) results in resistance to ciprofloxacin at dilutions from 4 - 16 ug/ml (Charvalos, et al., 1996; Zirnstein, et al., 1999). Such mutations can be detected using molecular techniques such as mismatch amplification (Charvalos, et al., 1996), nonradioactive single-stand conformation polymorphism (Zirnstein, et al., 1999), and fluorogenic polymerase chain reaction (PCR; Wilson, et al., 2000).

Fluorogenic PCR offers several advantages over other molecular tests for identification of mutations. The process uses a non-extendable oligonucleotide hybridization probe that contains a fluorescent reporter dye and a quencher dye. During PCR cycling, the probe hybridizes to the template and is digested by the exonuclease activity of Taq DNA polymerase as it moves along the template strand. This cleavage results in an increase of fluorescent emission of the reporter, which can be measured by fluorescence spectrometry. Allelic discrimination testing can be done with fluorogenic PCR, using probes and reporter dyes specific to the target allele (mutant or wild type), and examination of the post-PCR fluorescence emission spectrum(Lee, et al., 1993). This assay can detect a single base mismatch in probes 20 to 30 nucleotides long (Livak, 1999). The advantages of this real-time PCR allelic discrimination assay are: 1) it employs a closed tube system, which reduces contamination of DNA; 2) the analysis is conducted in log phase while PCR product is increasing (as opposed to end point assay in plateau phase when PCR product concentration is stationary), allowing more than one

gene to be analyzed simultaneously; 3) there is no post-PCR processing step, which saves time, labor and resources; and 4) it is compatible with automated technologies (Heid, et al., 1996).

Isolation, identification and *in vitro* susceptibility test of *C.jejuni* is laborious and time consuming. In order to conduct large scal epidemiological study or surveillance of fluoroquinolone resistance *C.jejuni*, a diagnostic tool allowing rapid process of the sample and compatible with with automation technologies is desirable. Because of the advantages that fluorogenic PCR can offer to epidemiological study and surveillance of fluoroquinolone resistance *C.jejuni*, it was decided to exploit the potentials of this assay to study *C. jejuni* isolates obtained from a country where fluoroquinolone resistance was prevalent. We hypothesize that the Thr-86 mutation in the *gyrA* gene of *C. jejuni* can be observed in field isolates, and that the presence of this mutation is correlated with resistance to ciprofloxacin and nalidixic acid. The objectives of our study were to: 1) determine the frequency of the Thr-86 mutation in *gyrA* in *C. jejuni* isolated from food animals and farm workers in northern Thailand; and 2) investigate the association between the Thr-86 mutation in *gyrA* and resistance to ciprofloxacin and nalidixic acid in *C. jejuni*.

MATERIAL AND METHODS

Source of *Campylobacter* isolates. The bacteria in this study were derived from a cross-sectional epidemiological study of *Campylobacter spp.* in food animals and farm workers in northern Thailand during the summer months of 2000 and 2001. A total of 84 isolates of *C. jejuni* were used in this study: 65 from samples from healthy chickens on

farms, 16 from chicken samples at slaughterhouse, one from chicken meat at the market, and one isolate from a healthy farm worker.

Isolation and identification of *Campylobacter spp.* Isolation of *Campylobacter spp.* was achieved using enrichment media (Bolton broth) and selective agar (Karmali or Preston agar). Suspected *Campylobacter* colonies were confirmed by oxidase test (Dryslide, BBL), catalase test (3% H_2O_2), and gram stain. Gram-negative spiral rods that were oxidase and catalase positive were identified as *Campylobacter*, frozen and stored in 30% glycerol with Mueller-Hinton broth at -70°C. The stock bacteria were transported from Thailand to Michigan State University (MSU) where in vitro susceptibility testing was conducted. Speciation of *C. jejuni* was done using 5'-nuclease fluorogenic PCR assay (Padungtod, et al., 2002) conducted at MSU.

In vitro susceptibility testing . In vitro susceptibility testing was done using the microbroth dilution method, following guidelines provided by the National Committee on Clinical Laboratory Standards (NCCLS)(NCCLS, 1997). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 37°C under microaerophilic conditions (85%N_2, 5% CO_2, 10% O_2). Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were scraped from the BASB with a sterile cotton swab and suspended in 5 ml H_2O. The turbidity was adjusted to a 0.5 McFarland standard using a standard solution, and one ml of the bacterial suspension was then added to 9 ml of Haemophilus testing medium (HTM). The final concentration of the inoculum was approximately 8 x 10^5 CFU/ml.

Customized SensiTitre plates were purchased pre-made from TREK Diagnostic Systems, Inc., with azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline. *C. jejuni* ATCC33560 was used as a quality control strain since it was shown to produce repeatable antimicrobial resistance profiles in microbroth dilution testing. Each plate was inoculated by adding 100 ul of the bacterial suspension using an autoinoculator, covered with a gas-permeable seal, and incubated at 42EC at 5% CO_2 for 48 hours. The minimum inhibitory concentration (MIC) was identified by the minimum dilution at which no bacterial growth occurred. The breakpoints used to categorize isolates as resistant or not resistant ($\geq$ 4 :g/ml for ciprofloxacin and $\geq$ 32 :g/ml for nalidixic acid) were those recommended by the National Antimicrobial Resistance Monitoring System (NARMS).

Fluorogenic PCR assay. A previous report describes identification of fluoroquinolone-resistant *C. jejuni* by use of a fluorogenic PCR, which discriminates between wild-type *C. jejuni* (susceptible to fluoroquinolone) and *C. jejuni* strains with mutation in codon 86 of the *gyrA* gene (Wilson, et al., 2000). The DNA of *C. jejuni* were extracted using QIAquick column (Qiagen), following manufacturer's recommendations. Final DNA products were diluted in sterile water to achieve a concentration of 1 :g/ml. In brief, primers JL238 and JL239, along with probes TAQ2 and TAQ3, were used in 50 (l PCR system. The fluorogenic assay PCR reaction mix contained the 1X Taqman buffer (PE Applied Biosystem, Branchburg, NJ), 0.2 mM of each dNTP (0.4 mM dUTP), 0.5 pmol of each primer/mL, 200 nM of each fluorogenic probe, 0.05 U of Amplitaq Gold polymerase (Perkins-Elmer)/mL, 0.01 U of Amperase UNG (Perkins-Elmer)/mL, 4.5 mM $MgCl_2$, 0.05% gelatin, and 0.01% Tween20. Each reaction mix contained 10 ng of

chromosomal DNA. Initial denaturation was conducted at 95°C for 10 min, the annealing and polymerization steps were combined at 60°C for 1 min, followed by denaturation at 95°C for 30 seconds. This process was cycled 40 times. Fluorescence emission detection and allelic discrimination was done using an ABI Prism 7700 Sequence Detection System (Perkins-Elmer). The wild-type control strain used was *C. jejuni* 81176, and a laboratory strain of *C. jejuni* 81176 with a ciprofloxacin MIC of 16 :g/ml (which carry the Thr-86-to-Ile mutation in *gyrA*) was used as the mutant control strain.

Statistical analysis. A Chi-square test was used to determine the significance level of association between the proportion of *C. jejuni* with the Thr-86-to-Ile mutation and ciprofloxacin or nalidixic acid resistance. The kappa statistic was computed to indicate the level of agreement between resistance categorization using MIC values and the presence of Thr-86-to-Ile mutation.

RESULTS

Of the 84 *C. jejuni* isolates tested, 60 were found to have the Thr-86-to-Ile mutation by fluorogenic PCR assay (Table 7-1). All *C. jejuni* isolates with ciprofloxacin MIC values > 2 :g/ml carried Thr-86-to-Ile mutation. When categorized by resistance, all *C. jejuni* with resistance to ciprofloxacin carried the mutation (Figure 7-1). However, there was one isolate of *C. jejuni* with no resistance to ciprofloxacin (MIC = 2:g/ml) that carried the mutation. All *C. jejuni* isolates with a nalidixic acid MIC below 16 :g/ml did not carry the Thr-86-to-Ile mutation. Of the 62 *C. jejuni* isolates with nalidixic acid MIC

≥16 :g/ml, two did not carry the mutation (Figure 7-2). When categorized by resistance, all *C. jejuni* with resistance to nalidixic acid (MIC ≥32 :g/ml) carried the mutation.

There was very high agreement between ciprofloxacin resistance and the presence of the Thr-86-to-Ile mutation (Table 7-2). The level of agreement was lower for nalidixic acid resistance categories.

DISCUSSION AND CONCLUSION

The fluorogenic PCR assay for allelic discrimination used in our study has been shown to be very specific, and faster than regular PCR or gene sequencing (Wilson, et al., 2000). Assays for identification of *C. jejuni* could be performed using whole bacteria, which would bypass the DNA extraction step (Padungtod, et al., 2002) and further speed the process, but conditions for the PCR may require further optimization to take advantage of this.

Previous studies have demonstrated the Thr-86-to-Ile mutation in *C. jejuni* in human clinical isolates (Gibreel, et al., 1998; Ruiz, et al., 1998; Zirnstein, et al., 1999). Our study obtained *Campylobacter* from healthy animals, which may better represent the general *C. jejuni* population at large. This study indicates that the Thr-86-to-Ile mutation may be more prevalent in general *C. jejuni* population than previously report (Wilson, et al., 2000).

The Thr-86 mutation in *C. jejuni* has been associated with ciprofloxacin resistance (MIC ≥ 4 :g/ml ; Zirnstein, et al., 1999). Our study found this mutation in *C. jejuni* with ciprofloxacin MIC values as low as 2 :g/ml. This may be due to the fact that isolates included in these studies were collected from sources that were not comparable (clinical

isolates versus isolates from healthy animals. The MIC values were generated using different techniques (agar dilution (Ruiz, et al., 1998; Zirnstein, et al., 1999) versus microbroth dilution in this study), which may result in different MIC values for the same bacteria (Caprioli, et al., 2000). Finally, there may be other mechanisms involved in reducing the susceptibility of *C. jejuni* to fluoroquinolones, including mutations in parC gene (Gibreel, et al., 1998) or the efflux pump (Charvalos, et al., 1995). Sequencing the genes of our *C. jejuni* isolates may clarify which additional mechanism(s) are involved in reducing fluoroquinolone susceptibility in *C. jejuni* isolated from food animals in Thailand.

The presence of Thr-86-to-Ile mutation correlated very well with categorization of *C. jejuni* using NARMS breakpoints for ciprofloxacin and nalidixic acid. As this study demonstrated, the assay has a potential to be used as a screening tool for detecting fluoroquinolone resistance in *C. jejuni* in samples from animals, humans, and food. Such a screening tool will be very useful in areas where fluoroquinolones resistance *C. jejuni* as a result of Thr-86-to-Ile mutation is prevalent.

In summary, our study showed high prevalence of *C. jejuni* with the Thr-86-to-Ile mutation in the *gyrA* gene. This mutation confers resistance to ciprofloxacin at MIC $\geq$ 4 μg/ml, and correlates very well with resistance categories defined by the NARMS breakpoints.

Table 7-1 MIC levels and mutations in *gyrA* gene in *C. jejuni*

Ciprofloxacin MIC (μg/ml)	Nalidixic acid MIC (μg/ml)	Number tested	Number with mutation
0.03	2	2	0
	4	3	0
	8	1	0
0.06	4	7	0
	64	1	0
0.12	4	6	0
	8	2	0
	16	1	0
0.25	4	1	0
2	64	1	1
4	16	3	3
	32	5	5
	64	7	7
	128	1	1
8	32	4	4
	64	21	21
	128	5	5
16	16	1	1
	64	4	4
	128	7	7
64	64	1	1
Total		84	60

Table 7-2 Proportion of *C. jejuni* with mutation and resistance

Antimicrobial agents	**Ciprofloxacin**	**Nalidixic acid**
Number tested	84	84
Number with resistance determined by *in vitro* susceptibility test	59	57
Number of resistant *C. jejuni* with Thr-86 mutation	59	56
Number of resistant *C.jejuni* without Thr-86 mutation	24	23
Sensitivity (%)	100	98.2
Specificity (%)	96	70
Chi-square p value	< 0.01	< 0.01

Figure 7-1 Number of *Campylobacter* isolates with the Thr-86-to-Ile mutation by MIC for ciprofloxacin (total 84 isolates, 60 with mutation)

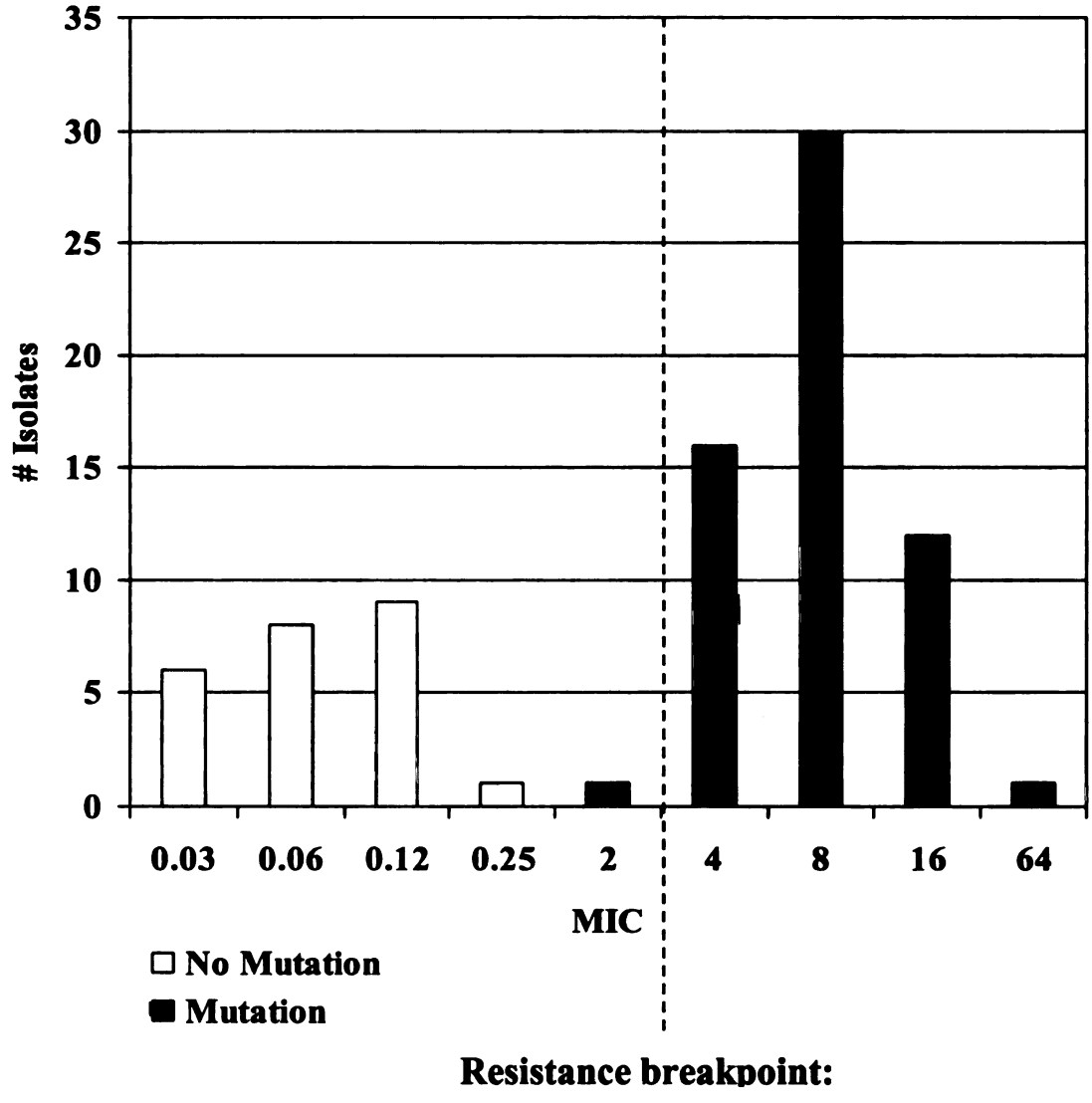
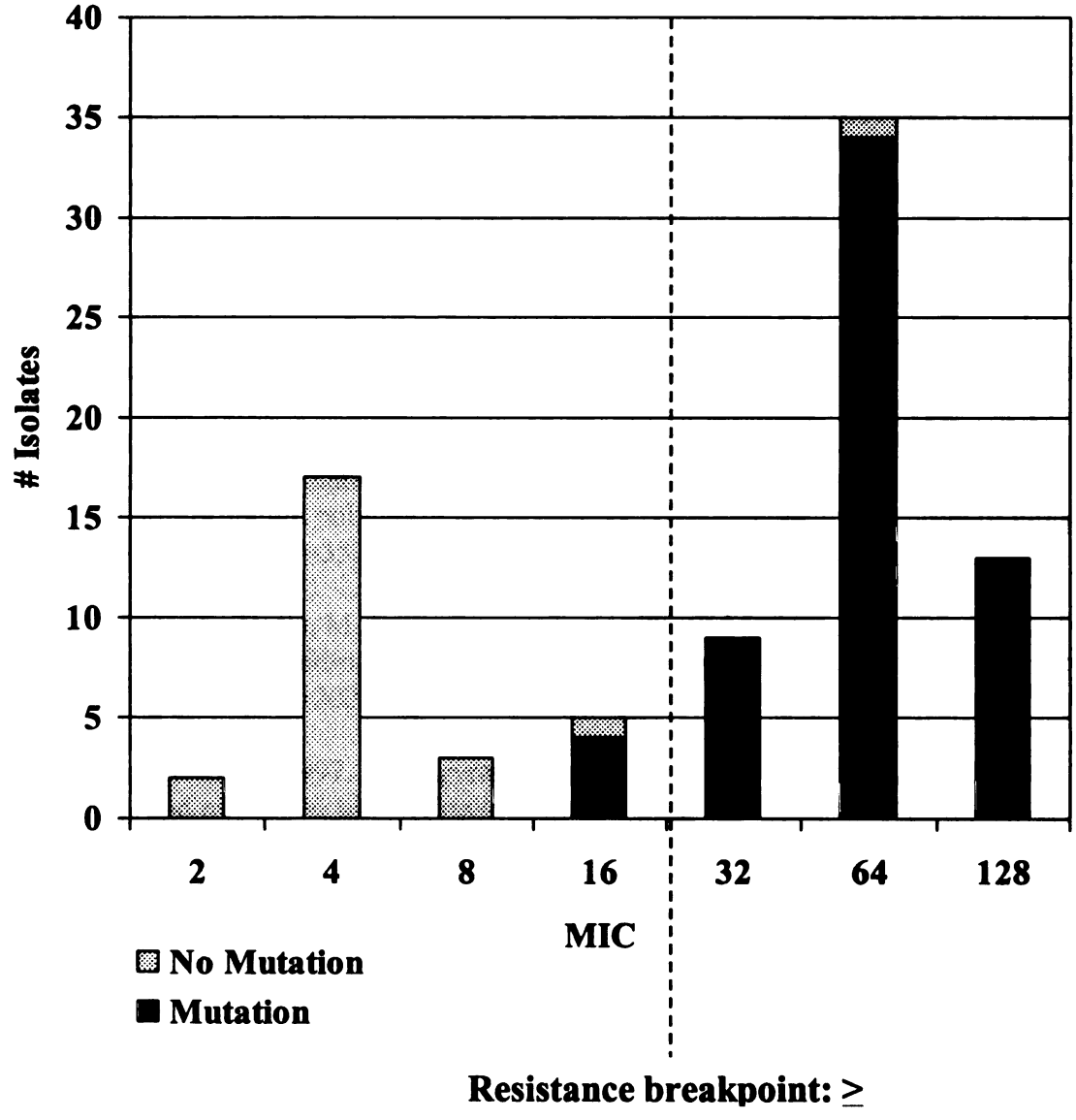


Figure 7-2 Number of *Campylobacter* isolates with the Thr-86-to-Ile mutation by MIC for nalidixic acid (total 84 isolates, 60 with mutation)



REFERENCES

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Atanassova, V., and C. Ring. 1999. Prevalence of *Campylobacter spp.* in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Berends, B., A. Van Den Bogaard, F. Van Knapen, and J. Snijders. 2001. Human health hazards associated with the administration of antimicrobials to slaughter animals: Part 2 An assessment of the risks of resistance bacteria in pigs and porks. Vetrinary quarterly. 23:10-21.

Caprioli, A., L. Busani, J. Martel, and R. Helmuth. 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. International Journal of antimicrobial agents. 14:295-301.

Charvalos, E., E. Peteinaki, I. Spyridaki, S. Manetas, and Y. Tselentis. 1996. Detection of ciprofloxacin resistance mutations in *Campylobacter jejuni gyrA* by nonradioisotopic single strand conformation polymorphism and direct DNA sequencing. Journal of clinical laboratory analysis. 10:129-33.

Charvalos, E., Y. Tselentis, M. Hamzehpour, T. Kohler, and J. Pechere. 1995. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni.* Antimicrobial agents and chemotherapy. 39(9):2019-2022.

Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiology and molecular biology review. 61:377-392.

Gibreel, A., E. Sjogren, B. Kaijser, B. Wretlind, and O. Skold. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC.* Antimicrobial agents and chemotherapy. 42(12):3276-3278.

Hart, C., and S. Kariuki. 1998. Antimicrobial resistance in developing countries. British medical journal. 317:647-650.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Genome research. 6:986-994.

Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsri, and P. Echeverria. 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clinical infectious diseases. 26:341-345.

Isenbarger, D., C. Hoge, A. Srijan, C. Pitarangsi, N. Vithayasai, L. Bodhidatta, K. Hickey, and P. Cam. 2002. Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996-1999. Emerging infectious diseases. 8(2):175-180.

Jacob-Reitsma, W. F., C. A. Kan, and N. M. Bolder. 1994. The induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment. Letters in applied microbiology. 19:228-231.

Lee, C. Y., C. L. Tai, S. C. Lin, and Y. T. Chen. 1994. Occurence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. International journal of food microbiology. 24:161-170.

Lee, L. G., C. R. Connel, and W. Bloch. 1993. Allelic discrimination by nick translation PCR with fluorogenic probes. Nucleic acid research. 21:3761-3766.

Livak, K. J. 1999. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genetic analysis: biomolecular engineering. 14:143-149.

McDermott, P., S. Bodeis, L. English, D. White, R. Walker, S. Zhao, S. Simjee, and D. Wagner. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. The journal of infectious diseases. 185:837-840.

NCCLS. 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (M37-A4), 4 ed. NCCLS, Wayne, PA.

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Ruiz, J., P. Goni, F. Marco, F. Gallardo, B. Mirelis, T. Jimenez-de-Anta, and J. Vila. 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates. Microbiology and immunology. 42(3):223-226.

Wang, Y., W. M. Huang, and D. E. Taylor. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutation. Antimicrobial agents and chemotherapy. 37(3):457-463.

Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz. 2000. Identification of Ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. Journal of clinical microbiology. 38(11):3971-3978.

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. Science. 279:996-7.

Zirnstein, G., Y. Li, B. Swaminathan, and F. Angulo. 1999. Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyrA* resistance mutation by mismatch amplification mutation assay PCR and DNA sequence analysis. Journal of clinical microbiology. 37(10):3276-3280.

CONCLUSIONS

Results of work for each main objective:

1. Validate the use of a fluorogenic PCR assay to identify C. jejuni from field samples.

A rapid PCR-based 5' nuclease fluorogenic PCR assay for identifying *C. jejuni* was applied to isolates from chickens from farm and slaughterhouse in Thailand. This assay allowed identification of *C. jejuni* within one day after colonies appeared on selective media, and yielded results comparable to convention tests (kappa = 0.75). When using PCR-RFLP of 23S rRNA genes as a definitive confirmation of *C. jejuni,* the assay was able to identify more isolates correctly than conventional test kits.

2. Determine the frequencies and the antimicrobial susceptibility level of Campylobacter spp. isolated from food animals and workers at pig and chicken farms, slaughterhouses, and markets.

The prevalence of *Campylobacter spp.* in pigs in northern Thailand were 70.3%, 45.6% and 20.9% at the farms, slaughterhouses, and markets, respectively. In chickens, the prevalence of *Campylobacter spp.* were 73.4%, 50.6%, and 47.2% at farms, slaughterhouses, and markets, respectively. The prevalence of *Campylobacter spp.* with resistance to fluoroquinolones was higher than other antimicrobial agents in both pigs and chickens. Multi-resistant *Campylobacter spp.* (resistance to more than one agent) were seen in 79-97% of isolates from chickens, 96-100% in pigs, and 100% in farm workers. The three antimicrobial agents to which *Campylobacter* isolates from pigs and chickens

showed the highest levels of resistance were tetracycline, nalidixic acid, and ciprofloxacin in. Human isolates were also resistant to these three agents.

3. Compare the frequencies of Campylobacter spp. with resistance to antimicrobial agents in food animals, food products and farm workers.

Resistance to all eight antimicrobial agents tested were observed in *Campylobacter spp.* isolated from pigs, while resistance to six agents were observed in isolates from chickens and three agents in isolates from humans. No resistance to chloramphenicol and gentamycin were found in *Campylobacter spp.* isolated from chickens. Resistant to erythromycin, azithromycin and clindamycin was more prevalent in *Campylobacter spp.* isolated from pigs than from chickens. The overall antimicrobial resistance profiles were generally similar among *Campylobacter spp.* isolated from farms, slaughterhouses and market in both pigs and chickens.

4. Determine the risk factors associated with the observed frequencies of Campylobacter spp. and frequencies of Campylobacter spp. with resistance to antimicrobial agents isolated from various sources.

Smaller flock size of chickens and herd size of pig were significantly associated with reduced probability of finding *Campylobacter spp.*, but the magnitude of the effect was low (95% CI for OR 0.97-1.0). Older chickens were less likely to be infected with *Campylobacter spp.* than younger birds (OR=0.90).

5. Determine whether antimicrobial use in feed and treatment on pig and chicken farms is associated with the frequency of antimicrobial resistance in Campylobacter.

On both pigs and chickens farms, the prevalence of resistant *Campylobacter* was higher for those antimicrobial agents that were used on the farm in feed and/or for treatment. No resistance or extremely low frequency of resistance was observed for those antimicrobial agents not used on the farms.

6. Determine the association between mutation in gyrA gene of Campylobacter jejuni and ciprofloxacin resistance.

The fluorogenic PCR developed in the laboratory at MSU can be used to detect the Thr-86-to-Ile mutation in *gyrA* gene in *C. jejuni* from field samples. This mutation is associated with decreased susceptibility to ciprofloxacin (MIC $\geq$ 2 μg/mL)

The work presented in this thesis was carried out in the field in Thailand, and in laboratories in Thailand and the U.S.. This study has shown high prevalence of *Campylobacter spp.* in food animals through the production system in northern Thailand. High proportions of these *Campylobacter spp.* were resistant to antimicrobial agents commonly used for treatment of gastrointestinal infection in humans, including ciprofloxacin and erythromycin. Most importantly, *Campylobacter spp.* isolated from humans were resistant to the same agents that showed the highest prevalence of resistance in isolates from food animals. This suggests that antimicrobial resistance in *Campylobacter* isolates from humans is associated with the development of resistant bacteria in food animals exposed to antimicrobial agents.

This study had several major strengths. We were able to follow the same animals from the farm to slaughter and eventually to market during the second year of study, which provided a strong basis for comparison of prevalence among different sampling points. The use of enrichment media and microbroth dilution susceptibility testing enhanced the validity of the study by improving the detectability level of the testing protocol, and provided precise measurements of susceptibility. Enhancing the ability to detect *Campylobacter* from field samples improved the estimates of prevalence throughout the study. Reporting MIC values from antimicrobial susceptibility testing provided greater detail for detecting changes in susceptibility, rather than the simple classification of isolates as resistant or non-resistant. The weaknesses of the study were the limited number of farms and slaughterhouses participating in the study, and the lack of precise measurement of antimicrobial use on the farm. The limited number of facilities used was due to the difficulty in setting up an adequate animal tracking system throughout the production system. This limited the power of the study to clarify factors associated with the observed prevalence, however it was felt that this weakness would be made up in the improved animal tracking through the facilities that did participate in the study. The lack of precise measurement of antimicrobial use on the farms is an inherent problem on virtually all farms in developing countries, and limited the power of the study to determine the association between antimicrobial use and the prevalence of *Campylobacter spp.* with resistance to antimicrobial agents.

Although there were some limitations in this study, the information generated will contribute to the global picture of the epidemiology of *Campylobacter spp.* and

antimicrobial resistance problems in developing countries. A more intensive study to assess factors contributing to the development of *Campylobacter spp.* with resistance to antimicrobial agents in developing countries, as well as further intervention studies comparing resistance in farms that do and do not use antimicrobial agents, should be conducted in the near future.

APPENDICES

APPENDIX 1

DATA COLLECTION FORM FOR PIG FARMS

Farm name: _____

Address: _____

Telephone _____

Contact person _____

Number of pigs:

Sows	Boars	Piglets	Fattening	Other	**Total**

Number of houses _____

Number of pigs per house _____

Number of workers per house _____

Use _____farm mix feed _____ buy ready mix product

Use growth promoter/ probiotic/ prophylactic antibiotic during the past 12 month

_____No

_____Yes,

Product name	*Amount*	*Type of Abx*	*Group of animal*	*Remark*

APPENDIX 2

SAMPLE COLLECTION FORM FOR PIGS AT FARMS

Farm name_____Date_____

Sample #	*House #*	*Pen #*	*Age*	*Breed*	*Remark*

APPENDIX 3

SAMPLE COLLECTION FORM FOR WORKERS AT PIG FARMS

Farm name_____ Date_____

Sample #	*House #*	*Age (year)*	*Sex*	*Abx use during previous month*

APPENDIX 4

DATA COLLECTION FORM FOR CHICKEN FARMS

Farm name _____
Address _____
Telephone _____

Contact person _____

Number of birds:

Broilers	*Number*
Week 1	
Week 2	
Week 3	
Week 4	
Week 5	
Week 6	
Week 7	
Other	
Total	

Number of houses_____
Number of birds per house _____
Number of workers per house _____

Use _____farm mix feed _____ buy ready mix product
Use growth promoter/ probiotic/ prophylactic antibiotic during the past 12 month
_____No
_____Yes,

Product name	*Amount*	*Type of Abx*	*Group of animal*	*Remarks*

APPENDIX 5

SAMPLE COLLECTION FORM FOR CHICKENS AT FARMS

Farm name_____Date_____

Sample #	*House #*	*Room #*	*Age*	*Breed*	*Type*	*Remarks*

APPENDIX 6

SAMPLE COLLECTION FORM FOR WORKERS AT CHICKEN FARMS

Farm name_____Date_____

Sample #	*House #*	*Age (year)*	*Sex*	*Abx use during previous month*

REFERENCES

REFERENCES

Aarestrup, F., and J. Engberg. 2001. Antimicrobial resistance of thermophilic *Campylobacter*. Veterinary research. 32:311-321.

Aarestrup, F., A. Seyfarth, H. Emborg, K. Pedersen, R. Hendriksen, and F. Bager. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal *Enterococci* from food animals in Denmark. Antimicrobial agents and chemotherapy. 45(7):2054-2059.

Acheson, D. 2001. Foodborne diseases update: current trends in foodborne diseases. Medscape Infectious diseases. 4(10):1017.

Allos, B. 2001. *Campylobacter jejuni* Infections: Update on emerging issues and trends. Clinical infectious diseases. 32:1201-1206.

Altekruse, S., D. Swerdlow, and N. Stern. 1998. *Campylobacter jejuni*. Veterinary clinics of North America: Food animal practice. 14(1):31-40.

Anderson, S. A., Y. Woo, and L. M. Crawford. 2001. Risk assessment of the impact on human health of resistant *Campylobacter jejuni* from fluoroquinolone use in beef cattle. Food control. 12(1):13-25.

Atanassova, V., and C. Ring. 1999. Prevalence of *Campylobacter spp.* in poultry and poultry meat in Germany. International journal of food microbiology. 51:187-190.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1997. Current protocols in molecular biology, vol. 1-3. John Wiley & son, New York, NY.

Barton, M. 1998. Does the use of antibiotics in animals affect human health? Australian veterinary journal. 76(3):177-180.

Bager, F., M. Madsen, J. Christensen, F.M. Aarestrup. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Preventive veterinary medicine. 31:95-112.

Berends, B., A. Van Den Bogaard, F. Van Knapen, and J. Snijders. 2001. Human health hazards associated with the administration of antimicrobials to slaughter animals: Part 2 An assessment of the risks of resistance bacteria in pigs and porks. Vetrinary quarterly. 23:10-21.

Berndtson, E., M. Danialsson-Tham, and A. Engvall. 1996. *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. International journal of food microbiology. 32:35-47.

Berndtson, E., U. Emanuelson, A. Engvall, and M. L. Danielsson-Tham. 1996. A 1-year epidemiological study of *Campylobacter* in 18 Swedish chicken farms. Preventive veterinary medicine. 26:167-185.

Berrang, M., and J. Dickens. 2000. Presence and level of *Campylobacter spp.* on broiler carcasses throughout the processing plant. Journal of applied poultry research. 9:43-47.

Berrang, M., S. Ladely, and R. Buhr. 2001. Presence and level of *Campyobacter*, coliforms, *Escherichia coli*, and total *aerobacteria* recovered from broiler parts with and without skin. Journal of food protection. 64(2):184-188.

Berrang, M. E., R. J. Buhr, and J. A. Cason. 1999. *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. Poultry science. 79:286-290.

Blaser, M. 1997. Epidemiology and clinical features of *Campylobacter jejuni* infections. The journal of infectious diseases. 176(suppl 2):s103-s105.

Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. W. Dillen, and J. Woordaa. 1990. Rapid and simple method for purification of nucleic acids. Journal of clinical microbiology. 28(3):495-503.

Boonmar, S., A. Bangtrakulnonth, S. Pornruangwong, S. Samosornsuk, K. Kaneko, and M. Ogawa. 1998. Significant increase in antibiotic resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. Veterinary microbiology. 62:73-80.

Boonmar, S., N. Marnrin, S. Pornruangwong, and A. Bangtrakulnonth. 1997. Contamination of *Salmonella* in Chicken and pork products. Kasetsart journal of natural science. 31(4):413-418.

Busato, A., T. Lentze, D. Hofer, A. Burnens, B. Hentrich, and C. Gaillard. 1998. A case-control study of potential enteric pathogens for calves raised in cow-calf herds. Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 45:519-528.

Caprioli, A., L. Busani, J. Martel, and R. Helmuth. 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. International Journal of antimicrobial agents. 14:295-301.

Carvalho, A., G. Ruiz-Palacios, P. Ramos-Cervantes, L. Cervantes, X. Jiang, and L. Pickering. 2001. Molecular characterization of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. Journal of clinical microbiology. 39(4):1353-1359.

Casademont, I., C. Bizet, D. Chevrier, and J. Guesdon. 2000. Rapid detection of *Campylobacter fetus* by polymerase chain reaction combined with non-radioactive hybridization using an oligonucleotide covalently bound to microwells. Molecular and cellular probes. 14:233-240.

Cawthraw, S., L. Lind, B. Kaijser, and D. Newell. 2000. Antibodies, directed towards *Campylobacter jejuni* antigens, in sera from poultry abattoir workers. Clinical and experimental immunology. 122:55-60.

Charvalos, E., E. Peteinaki, I. Spyridaki, S. Manetas, and Y. Tselentis. 1996. Detection of ciprofloxacin resistance mutations in *Campylobacter jejuni* gyrA by nonradioisotopic single strand conformation polymorphism and direct DNA sequencing. Journal of clinical laboratory analysis. 10:129-33.

Charvalos, E., Y. Tselentis, M. Hamzehpour, T. Kohler, and J. Pechere. 1995. Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrobial agents and chemotherapy. 39(9):2019-2022.

Christensen, H., K. Jorgensen, and J. E. Olsen. 1999. Differentiation of *Campylobacter coli* and *C.jejuni* by length and DNA sequence of the 16S-23S rRNA internal spacer region. Microbiology. 145:99-105.

Chuma, T., S. Hashimoto, and K. Okamoto. 2000. Detection of thermophilic *Campylobacter* from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with *Campylobacter*. Journal of veterinary medical science. 62(12):1291-1295.

Chuma, T., T. Ikeda, T. Maeda, H. Niwa, and K. Okamoto. 2001. Antimicrobial susceptibilities of *Campylobacter* strains isolated from broilers in southern part of Japan from 1995-1999. Journal of veterinary medical science. 63(9):1027-1029.

Chuma, T., K. Makino, K. Okamoto, and H. Yugi. 1997. Analysis of distribution of *Campylobacter jejuni* and *Campylobacter coli* in broiler by using restriction fragment polymorphism of flaggelin gene. Journal of veterinary medical scienes. 59(11):1011-1015.

Chuma, T., K. Yano, H. Omori, K. Okamoto, and H. Yugi. 1997. Direct detection of *Campylobacter jejuni* in chicken cecal contents by PCR. Journal of veterinary medical science. 59(1):85-87.

Cockerill III, F. 1999. Genetic methods for assessing antimicrobial resistance. Antimicrobial agents and chemotherapy. 43(2):199-212.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of *Campylobacters*. International journal of food microbiology. 26(1):43-76.

Craven, S., N. Stern, E. Line, J. Bailey, N. Cox, and P. Fedorka-Cray. 2000. Determination of the incidence of *Salmonella spp.*, *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal dropping. Avian diseases. 44:715-720.

Cudjoe, K. S., and R. Krona. 1997. Detection of *Salmonella* from raw food samples using Dynabeads anti-Salmonella and a conventional reference method. International journal of food microbiology. 37(1):55-62.

Das, S. C., G. B. Nair, S. G. Mullickk, G. Biswas, A. Sikdar, and D. Bhatacharya. 1996. Study on in vitro antimicrobial sensitivity of *Campylobacter* species of animal and human origin. Indian journal of animal health. 35(2):193-196.

De Boer, P., B. Duim, A. Rigter, J. Van Der Plas, W. Jacobs-Reitsman, and J. Wagenaar. 2000. Computer assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. Journal of clinical microbiology. 38(5):1940-1946.

Deming, M., R. Tauxe, P. Blake, S. Dixon, B. Fowler, S. Jones, E. Lockamy, C. Patton, and R. Sikes. 1987. *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. American journal of epidemiology. 126(3):526-534.

Denis, M., C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat, and P. Colin. 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *Campylobacter coli*. Letters in applied microbiology. 29:406-410.

Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiology and molecular biology review. 61:377-392.

Duffy, E., K. Belk, J. Sofos, G. Bellinger, A. Pape, and G. Smith. 2001. Extent of microbial contamination in United States pork retail products. Journal of food protection. 64(2):172-178.

Dufrenne, J., W. Ritmeester, E. Delfgou-van-Asch, F. Leusden, and R. Jonge. 2001. Quantification of contamination of chicken and chicken products in the Netherlands with *Salmonella* and *Campylobacter*. Journal of food protection. 64(4):538-541.

Duim, B., C. Ang, A. Van Belkum, A. Rigter, N. Van Leeuwen, H. Endtz, and J. Wagenaar. 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barre or Miller-Fisher syndrome. Applied and environmental microbiology. 66(9):3917-3923.

Effler, P., M. Ieong, A. Kimura, M. Nakata, R. Burr, E. Cremer, and L. Slutsker. 2001. Sporadic *Campylobacter jejuni* infections in Hawaii: Association with prior antibiotic use and commercially prepared chicken. The journal of infectious diseases. 183:1152-1155.

Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohaemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proceeding of the national academy of sciences. 97(7):2999-3003.

Endtz, H. P., R. P. Mouton, T. D. Reyden, G. J. Ruijs, M. Biever, and B. Klingeren. 1990. Fluoroquinolone resistance in *Campylobacter spp.* isolated from human stool and poultry product. The Lancet. 335:787.

Endtz, H. P., G. J. Rujis, B. Klingeren, W. H. Jansen, T. Reyden, and R. P. Mouton. 1991. Quinolone resistance in *campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. Journal of antimicrobial chemotherapy. 27:199-208.

Engberg, J., F. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C.coli*: resistance mechanisms and trends in human isolates. Emerging infectious diseases. 7(1):24-34.

Englen, M., and L. Kelly. 2000. A rapid DNA isolation procedure for the identification of *Campylobacter jejuni* by polymerase chain reaction. Letters in applied microbiology. 31:421-426.

Evans, M., R. Roberts, C. Ribeiro, D. Gardner, and D. Kembrey. 1996. A milk-borne *campylobacter* outbreak following an educational farm visit. Epidemiology and infections. 117:457-462.

Evans, S. J., and A. R. Sayers. 2000. A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. Preventive veterinary medicine. 46:209-223.

Eyers, M., S. Chapelle, G. V. Camp, H. Goosens, and R. D. Wachter. 1993. Discrimination among thermophilic *Campylobacter* species by polymerase chain reaction amplification of 23S rRNA gene fragment. Journal of clinical microbiology. 31(12):3340-3343.

Fayos, A., R. Owen, and M. Desai, Hernandez, J. 1992. Ribosomal RNA gene restriction fragment diversity amonst Lior biotypes and penner serotype of *Campylobacter jejuni* and *Campylobacter coli*. FEMS microbiology letters. 95:87-94.

Fermer, C., and E. O. Engvall. 1999. Specific PCR identification and differentiation of the thermophilic *Campylobacters*, *Campylobacter jejuni*, *C.coli*, *C.lari* and *C. upsaliensis*. Journal of clinical microbiology. 37(10):3370-3373.

Fitzgerald, C., L. Helsel, M. Nicholson, S. Olsen, D. Swerdlow, R. Flahart, J. Sexton, and P. Fields. 2001. Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. Journal of clinical microbiology. 39(7):2386-2390.

Fitzgerald, C., R. Owen, and J. Stanley. 1996. Comprehensive ribotyping scheme for heat stable serotype of *Campylobacter jejuni*. Journal of clinical microbiology. 34(2):265-269.

Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones. 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. Applied and environmental microbiology. 67(4):1429-1436.

Frei, A., D. Goldenberger, and M. Teuber. 2001. Antimicrobial susceptibility of intestinal bacteria from Swiss poultry flocks before the band of antimicrobial growth promoters. Systemic and applied microbiology. 24:116-121.

Friedman, C., J. Neimann, H. Wegener, and R. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121-138. In I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Fujita, M., S. Fujimoto, T. Morooka, and K. Amako. 1995. Analysis of strains of *Campylobacter fetus* by pulsed-field gel electrophoresis. Journal of clinical microbiology. 33(6):1676-1678.

Gallardo, F., J. Gascon, J. Ruiz, M. Corachan, T. Anta, and J. Vila. 1998. *Campylobacter jejuni* as a cause of traveler's diarrhoea: clinical features and antimicrobial susceptibility. Journal of travel medicine. 5:23-26.

Garcia, M., M. Eaglesome, and C. Rigby. 1983. *Campylobacter* important in veterinary medicine. Veterinary bulletin. 53(9):793-818.

Gaudio, P., P. Echeverria, C. Hoge, J. Pitarangsri, and P. Goff. 1996. Diarrhea among expatriate residents in Thailand: correlation between reduced *Campylobacter* prevalence and longer duration of stay. Journal of traveller medicine. 3:77-79.

Gaudreau, C., and H. Gilbert. 1998. Antimicrobial resistance of clinical strains of *Campylobacter jejuni* subsp. *jejuni* isolated from 1985 to 1997 in Quebec, Canada. Antimicrobial agents and chemotherapy. 42(8):2106-2108.

Gaunt, P. N., and L. J. V. Piddock. 1996. Ciprofloxacin resistant *Campylobacter spp.* in humans: an epidemiological and laboratory study. Journal of antimicrobial chemotherapy. 37:747-757.

Genigeorgis, C., M. Hassuneh, and P. Collins. 1986. *Campylobacter jejuni* infection on poultry farms and its effect on poultry meat contamination during slaughtering. Journal of food protection. 49(11):895-903.

Gibreel, A., E. Sjogren, B. Kaijser, B. Wretlind, and O. Skold. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. Antimicrobial agents and chemotherapy. 42(12):3276-3278.

Glaab, W. E. and T. R. Skopek. 1999. A novel assay for allelic discrimination that combines the fluorogenic 5' polymerase chain reaction (Taqman) and mismatch amplification mutation assay. Fundamental and molecular mechanism of mutagenesis. 430(1): 1-12.

Hald, B., E. Rattenborg, and M. Madsen. 2001. Role of batch depletion of broiler houses on the occurence of *Campylobacter spp.* in chicken flocks. Letters in applied microbiology. 32:253-256.

Hanninen, M., M. Niskanen, and L. Korhonen. 1998. Water as a reservoir for *Campylobacter jejuni* infection in cows studied by serotyping and pulsed-field gel electrophoresis (PFGE). Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 45:37-42.

Hanninen, M., S. Pajarre, M. Klossner, and H. Rautelin. 1998. Typing of human *Campylobacter jejuni* isolates in Finland by pulse-field gel electrophoresis. Journal of clinical microbiology. 36(6):1787-1789.

Harrington, C., F. Thomson-Carter, and P. Carter. 1999. Molecular epidemiological investigation of an outbreak of *Campylobacter jejuni* identifies a dominant clonal line within Scottish serotype HS55 populations. Epidemiology and infections. 122:367-375.

Hart, C., and S. Kariuki. 1998. Antimicrobial resistance in developing countries. British medical journal. 317:647-650.

Harvey, R., C. Young, R. Anderson, R. Droleskey, K. Genovese, L. Egan, and D. Nisbet. 2000. Diminution of *Campylobacter* colonization in neonatal pigs reared off-sow. Journal of food protection. 63(10):1430-1432.

Harvey, R. B., C. R. Young, R. L. Ziprin, M. E. Hume, K. J. Genovese, R. C. Anderson, R. E. Drolesky, L. H. Stanker, and D. J. Nisbet. 1999. Prevalence of *Campylobacter spp.* isolated from the intestinal tract of pigs raised in an integrated swine production system. Journal of the american veterinary medical association. 215(11):1601-1604.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Genome research. 6:986-994.

Heuer, O., K. Pedersen, J. Andersen, and M. Madsen. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Letters in applied microbiology. 33:269-274.

Hoar, B. R., E. R. Atwill, C. Elmi, W. W. Uterback, and A. J. Edmondson. 1999. Comparison of fecal samples collected per rectum and off the ground for estimation of environmental contamination attributable to beef cattle. American journal of veterinary research. 60(11):1352-1356.

Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsri, and P. Echeverria. 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clinical infectious diseases. 26:341-345.

Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceeding of the national academy of science USA. 88:7276-7280.

Hoorfar, J., P. Ahrens, and P. Radstrom. 2000. Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. Journal of clinical microbiology. 38(9):3429-3435.

Hoorfar, J., E. M. Nielsen, H. Stryhn, and S. Andersen. 1999. Evaluation of two automated enzyme-immunoassays for detection of thermophilic *campylobacters* in fecal samples from cattle and swine. Journal of microbiological methods. 38:101-106.

Hopkins, R., and A. Scott. 1983. Handling raw chicken as a source for sporadic *Campylobacter jejuni* infections. The journal of infectious diseases. 148(4):770.

Houng, H., O. Sethabutr, W. Nirdnoy, D. Katz, and L. Pang. 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. Diagnostic microbiology and infectious disease. 40:11-19.

Huang, M., C. Baker, S. Banerjee, and F. Tenover. 1992. Acuracy of the E test for determining antimicrobial susceptibilities of *Staphylococci*, *enterococci*, *Campylobacter jejuni*, and gram-negative bacteria resistant to antimicrobial agents. Journal of clinical microbiology. 30(12):3243-3248.

Hurtado, A., and R. J. Owen. 1997. A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. Journal of clinical microbiology. 35(9):2401-2404.

Isenbarger, D., C. Hoge, A. Srijan, C. Pitarangsi, N. Vithayasai, L. Bodhidatta, K. Hickey, and P. Cam. 2002. Comparative antibiotic resistance of diarrheal pathogens from Vietnam and Thailand, 1996-1999. Emerging infectious diseases. 8(2):175-180.

Jacob-Reitsma, W. 1995. *Campylobacter* bacteria in breeder flocks. Avian diseases. 39:355-359.

Jacob-Reitsma, W. 1997. Aspects of epidemiology of *Campylobacer* in poultry. Veterinary quarterly. 19(3).

Jacob-Reitsma, W., N. Bolder, and R. Mulder. 1994. Cecal carriage of *Campylobacter* and *Salmonella* in Dutch broiler flocks at slaughter: A one-year study. Poultry science. 73:1260-1266.

Jacob-Reitsma, W., P. Koenraad, N. Bolder, and R. Mulder. 1994. In vitro susceptibility of Campylobacter and *Salmonella* isolates from broilers to quinolones, ampicillin, tetracycline, and erythromycin. Veterinary quarterly. 16(4):206-208.

Jacob-Reitsma, W., A. Van de Giessen, N. Bolder, and R. Mulder. 1995. Epidemiology of *Campylobacter spp.* at two dutch broiler farm. Epidemiology and infections. 114:413-421.

Jacob-Reitsma, W. F., C. A. Kan, and N. M. Bolder. 1994. The induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment. Letters in applied microbiology. 19:228-231.

Jensen, B., and F. Aarestrup. 2001. Macrolide resistance in *Campylobacter coli* of animal origin in Denmark. Antimicrobial agents and chemotherapy. 45(1):371-372.

Jones, F., R. Axtell, D. Rives, S. Scheideler, F. Tarver, R. Walker, and M. Wineland. 1991. A survey of *Campylobacter jejuni* Contamination in modern broiler production and processing system. Journal of food protection. 54(4):259-262.

Kalman, M., E. Szollosi, B. Czermann, M. Zimanyi, S. Szekeres, and M. Kalman. 2000. Milkborne *Campylobacter* infection in Hungary. Journal of food protection. 63(10):1426-1429.

Kapperud, G., E. Skjerve, N. Bean, S. Ostroff, and J. Lassen. 1992. Risk factors for sporadic *Campylobacter* infections : result of a case-control study in Southeatern Norway. Journal of clinical microbiology. 30(12):3117-3121.

Kazwala, R., J. Collins, R. Crinion, and H. O'Mahony. 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commercial poultry production. The veterinary record. 126:305-306.

Koenraad, P. M. F. J., W. F. Jacob-Reitsma, T. V. D. Laan, R. R. Reumer, and F. M. Rombouts. 1995. Antibiotic susceptibility of *Campylobacter* isolates from sewage and poultry abattoir drain water. Epidemiology and infection. 115:475-483.

Konkel, M. E., S. A. Gray, B. J. Kim, S. G. Garvis, and J. Yoon. 1999. Identification of enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. Journal of clinical microbiology. 37(3):510-517.

Korolik, V., D. Friendship, T. Peduru-Hewa, D. Alfredson, B. Fry, and P. Coloe. 2001. Specific identification, grouping and differentiation of *Campylobacter jejuni* among thermophilic *campylobacters* using multiplex PCR. Epidemiology and infections. 127:1-5.

Korolik, V., L. Moorthy, and P. Coloe. 1995. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* strains by using Restriction endonuclease DNA profiles and DNA fragment polymorphisms. Journal of clinical microbiology. 33(5):1136-1140.

Korsak, N., G. Daube, Y. Ghafir, A. Chahed, S. Jolly, and H. Vindevogel. 1998. An efficient sampling technique used to detect four foodborne pathogens on pork and beef carcasses in nine Belgian abattoirs. Journal of food protection. 61(5):535-541.

Kuschner, R. A., F. A. Trofa, R. J. Thomas, C. W. Hoge, C. Pitarangsi, S. Amato, R. P. Olafson, P. Echeveria, J. C. Sadoff, and D. N. Taylor. 1995. Use of Azithromicin for the treatment of *Campylobacter* enteritis in travelers to Thailand, an area where Ciprofloxacin resistance is prevalent. Clinical infectious diseases. 21:536-541.

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

Lawson, A. J., M. S. Shafi, K. Pathak, and J. Stanley. 1998. Detection of *campylobacter* in gastroenteritis: comparison of direct PCR assay of faecal samples with selective culture. Epidemiology and infection. 121:547-553.

Lea, T., F. Vartdal, C. Davies, and J. Ugelstad. 1985. Magnetic monosized polymer particles for fast and specific fractionation of human mononuclear cells. Scandinavian journal of immunology. 22:207-216.

Lee, C. Y., C. L. Tai, S. C. Lin, and Y. T. Chen. 1994. Occurence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. International journal of food microbiology. 24:161-170.

Lee, L. G., C. R. Connel, and W. Bloch. 1993. Allelic discrimination by nick translation PCR with fluorogenic probes. Nucleic acid research. 21:3761-3766.

Lehner, A., C. Schneck, G. Feierl, P. Plees, A. Deutz, E. Brandl, and M. Wagner. 2000. Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of

Campylobacter jejuni in an Austrian youth center. Epidemiology and infections. 125:13-16.

Levy, S.B. 1987. Antibiotic use for growth promotion in animals: Ecologic and public health consequences. Journal of food protection. 50(7): 616-620.

Li, C. C., C. H. Chiu, J. L. Wu, Y. C. Huang, and T. Y. Lin. 1998. Antimicrobial susceptibilities of *Campylobacter jejuni* and *coli* by using E-test in Taiwan. Scandinavian journal of infectious disease. 30:39-42.

Lilja, L., and M. Hanninen. 2001. Evaluation of a commercial automated ELISA and PCR method for rapid detection and identification of *Campylobacter jejuni* and *C.coli* in poultry products. Food microbiology. 18:205-209.

Lindstedt, B., E. Heir, T. Vardund, K. Melby, and G. Kapperud. 2000. Comparative fingerprinting analysis of *Campylobacter jejuni* subsp. *jejuni* strains by amplified fragment length polymorphism genotyping. Journal of clinical microbiology. 38(9):3379-3387.

Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of clinical microbiology. 35(10):2568-2572.

Livak, K. J., S. J. A. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR methods and applications. 4:357-362.

Livak, K. J. 1999. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genetic analysis: biomolecular engineering. 14:143-149.

Logan, J., K. Edwards, N. Saunders, and J. Stanley. 2001. Rapid identification of *Campylobacter spp.* by melting peak analysis of biprobes in real time PCR. Journal of clinical microbiology. 36(6):2227-2232.

Lucey, B., C. Feurer, P. Greer, P. Moloney, B. Cryan, and S. Fanning. 2000. Antimicrobial resistance profiling and DNA Amplification fingerprinting (DAF) of thermophilic *Campylobacter spp.* in human, poultry and porcine samples from the Cork region of Ireland. Journal of applied microbiology. 89:727-734.

Ludwig, W., and K. Schleifer. 2000. How quantitative is quantitative PCR with respect to cell counts? Systemic and applied microbiology. 23:556-562.

Marshall, S., P. Melito, D. Woodward, W. Johnson, F. Rodgers, and M. Mulvey. 1999. Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-

Restriction Fragment Length Polymorphism analysis of 16S rRNA gene. Journal of clinical mocrobiology. 37(12):4158-4160.

Mazurier, S., S. Van de Geissen, K. Heuvelman, and K. Wernars. 1992. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Letters in applied microbiology. 14:260-262.

McDermott, P., S. Bodeis, L. English, D. White, R. Walker, S. Zhao, S. Simjee, and D. Wagner. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. The journal of infectious diseases. 185:837-840.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the united states. Emerging infectious diseases. 5(5):607-625.

Metherell, L., J. Logan, and J. Stanley. 1999. PCR enzyme link immunosorbent assay for detection and identification of *Campylobacter* species: Application to isolates and stool samples. Journal of clinical microbiology. 37(2):433-435.

Mirelis, B., T. Llovet, C. Munoz, F. Navarro, and G. Prats. 1999. Resistance of *Salmonella* and *Campylobacter* species to antimicrobial agents. European journal of clinical microbiology and infectious diseases. 18:312.

Misawa, N., S. Shinohara, H. Satoh, H. Itoh, K. Shinohara, K. Shimomura, F. Kondo, and K. Itoh. 2000. Isolation of *Campylobacter* species from zoo animals and polymerase chain reaction-based random amplified polymorphism DNA analysis. Veterinary microbiology. 71:59--68.

Moore, J. E., and P. G. Murphy. 2000. Inhibition of selective media in the isolation of thermophilic *Campylobacter spp.* from foods. British journal of biomedical science. 57:150-151.

Morgan, G., P. Chadwick, K. Lander, and K. Gill. 1985. Campylobacter jejuni mastitis in a cow: a zoonosis related incident. The veterinary record. 116(4):111.

Murphy Jr, G., P. Echeverria, L. Jackson, M. Arness, C. Lebron, and J. Pitarangsi. 1996. Ciprofloxacin and azithromycin resistant *Campylobacter* causing traveller's diarrhea in US troops deployed to Thailand in 1994. Clinical infectious diseases. 22:868-869.

Nachamkin, I. 1999. *Campylobacter* and *Arcobacter*, p. 716-726. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7 ed. ASM press, Washington DC.

Nachamkin, I., K. Bohachick, and C. Patton. 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. Journal of clinical microbiology. 31(6):1531-1536.

Nachamkin, I., J. Engberg, and F. M. Aaestrup. 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, p. 45-66. In I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

NCCLS. 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (M37-A4), 4 ed. NCCLS, Wayne, PA.

Neu, H.C. 1992. The crisis in antibiotic resistance. Science. 257:1064-1073.

Newell, D., J. Shreeve, M. Toszeghy, G. Dominigue, S. Bull, T. Humphrey, and G. Mean. 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. Applied and environmental microbiology. 67(6):2636-2640.

Oberhelman, R., and D. Taylor. 2000. Campylobacter infections in developing countries, p. 139-153. In I. Nachamkin and M. Blaser (ed.), Campylobacter, 2 ed. ASM press, Washington DC.

Olsen, S., G. Hansen, L. Bartlett, C. Fitzgerald, A. Sonder, R. Manjrekar, T. Riggs, J. Kim, R. Flahart, G. Pezzino, and D. Swerdlow. 2001. An outbreak of *Campylobacter jejuni* infections associated with food handler contamination: The use of pulse-field gel electrophoresis. The journal of infectious diseases. 183:164-167.

On, S. 1998. In vitro genotypic variation of Campylobacter coli documented by pulsed-field gel electrophoretic DNA profiling: implications for epidemiological studies. FEMS microbiology letters. 165:341-346.

On, S., and C. Harrington. 2001. Evaluation of numerical analysis of PFGE-DNA profiles for differentiating *Campylobacter fetus* subspecies by comparison with phenotypic, PCR and 16S rDNA sequencing methods. Journal of applied microbiology. 90:285-293.

On, S. L. W. 1996. Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. Clinical microbiology reviews. 9(3):405-422.

Ono, K., and K. Yamamoto. 1999. Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. International journal of food microbiology. 47:211-219.

Osano, O., and S. Arimi. 1999. Retail poultry and beef as sources of *Campylobacter jejuni*. East african medical journal. 76(3):141-143.

O'Sullivan, N. A., R. Fallon, C. Carroll, T. Smith, and M. Maher. 2000. Detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in broiler chicken samples using a PCR/DNA probe membrane based colorimetric detection assay. Molecular and cellular probes. 14(1):7-16.

Owen, R., M. Desai, and S. Garcia. 1993. Molecular typing of thermotolerant species of *Campylobacter* with ribosomal RNA gene patterns. Researh in microbiology. 144(709-720).

Padungtod, P., D. Wilson, J. Bell, J. Kaneene, R. Hanson, and J. E. Linz. 2002. Identification of *Campylobacter jejuni* isolates from chicken fecal and carcass by use of a fluorogenic PCR assay. Journal of food protection. In press.

Patton, C., I. Wachsmuth, G. Evins, J. Kiehlbauch, B. Plikaytis, N. Troup, L. Tompkins, and H. Lior. 1991. Evaluation of 10 methods to distinguish epidemic associated *Campylobacter* strains. Journal of clinical microbiology. 29(4):680-688.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by water borne *Campylobacter jejuni*. Applied and environmental microbiology. 59(4):987-996.

Pearson, A. D., M. H. Greenwood, J. Donaldson, T. D. Healing, D. M. Jones, M. Shahamat, R. K. A. Feltham, and R. R. Colwell. 2000. Continuous source outbreak of Campylobacteriosis traced to chicken. Journal of food protection. 63(3):309-314.

Pearson, A. D., M. H. Greenwood, R. K. A. Feltham, T. D. Healing, J. Donaldson, D. M. Jones, and R. R. Colwell. 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: Intermittent common source, vertical transmission, and amplification by flock propagation. Applied and environmental microbiology. 62(12):4614-4620.

Petersen, L., E. Nielsen, and S. On. 2001. Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. Veterinary microbiology. 82:141-154.

Petersen, L., and S. On. 2000. Efficacy of flagellin gene typing for epidemiological studies of *Campylobacter jejuni* in poultry estimated by comparison with macrorestriction profiling. Letters in applied microbiology. 31:14-19.

Phetsouvanh, R., Y. Midorikawa, and S. Nakamura. 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 journal of tropical medicine and public health. 30(2):319-323.

Piddock, L. J. V. 1996. Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic resistant bacteria that infect man and compromise antimicrobial chemotherapy. Journal of antimicrobial chemotherapy. 38(1):1-3.

Pigrau, C., R. Bartolome, B. Almirante, A. M. Planes, J. Gavalda, and A. Pahissa. 1997. Bacteremia due to *Campylobacter* species: clinical findings and antimicrobial susceptibility patterns. Clinical infectious diseases. 25:1414-1420.

Poocharoen, L., and C. Bruin. 1986. *Campylobacter jejuni* in hospitalized children with diarrhoea in Chiang Mai, Thailand. Southeast Asian journal of tropical medicine and public health. 17(1):53-58.

Prasad, K. N., S. K. Mathur, T. N. Dhole, and A. Ayyagari. 1994. Antimicrobial susceptibility and plasmid analysis of *Campylobacter jejuni* isolated from diarrhoeal patients and healthy chickens in Northern India. journal of diarrhoeal disease research. 12(4):270-273.

Prats, G., B. Mirelis, T. Llovet, C. Munoz, E. Miro, and F. Navarro. 2000. Antibiotic resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona. Antimicrobial agents and chemotherapy. 44(5):1140-1145.

Prescott, J. 2000. Lincosamides, macrolides, and pleuromutilins. In J. Prescott, J. Baggot, and R. Walker (ed.), Antimicrobial therapy in veterinary medicine, 3 ed. Iowa state university press, Ames.

Quinn, P., M. E. Carter, B. A. Markey, and G. R. Carter. 1994. Clinical veterinary microbiology. Wolfe publishing, London.

Radostits, O., C. Gay, D. Blood, and K. Hinchcliff. 2000. Veterinary Medicine, 9 ed. W.B.Saunders, London.

Rasmussen, H., J. Olsen, K. Jorgensen, and O. Rasmussen. 1996. Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken fecal sample by PCR. Letters in applied microbiology. 23:363-366.

Rasrinual, L., O. Suthienkul, P. Echeverria, D. Taylor, J. Seriwatana, A. Bangtrakulnonth, and U. Lexomboon. 1988. Foods as source of enteropathogens causing childhood diarrhea in Thailand. American journal of tropical medicine and hygiene. 39(1):97-102.

Rautelin, H., J. Jusufovic, and M. J. Hanninen. 1999. Identification of hippurate-negative thermophilic *Campylobacter*. Diagnostic microbiology and infectious diseases. 35(1):9-12.

Refregier-Petton, J., N. Rose, M. Denis, and G. Salvat. 2001. Risk factors for *Campylobacter spp.* contamination in French broiler-chicken flocks at the end of the rearing period. Preventive veterinary medicine. 50:89-100.

Ribot, E., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. Barrett. 2001. Rapid pulse field gel electrophoresis protocol for subtyping *Campylobacter jejuni*. Journal of clinical microbiology. 39(5):1889-1894.

Rivoal, K., M. Denis, G. Salvat, P. Colin, and G. Ermel. 1999. Molecular characterization of the diversity of *Campylobacter spp.* isolates collected from a poultry slaughterhouse: analysis of cross contamination. Letters in applied microbiology. 29:370-374.

Rosner, B. 1995. Fundamental of biostatistics, 4 ed. Duxbery press, New York.

Ruiz, J., P. Goni, F. Marco, F. Gallardo, B. Mirelis, T. Jimenez-de-Anta, and J. Vila. 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates. Microbiology and immunology. 42(3):223-226.

Saeed, A., N. Harris, and R. DiGiacomo. 1993. The role of exposure to animals in the etiology of *Campylobacter jejuni/coli* enteritis. American journal of epidemiology. 137(1):108-114.

Saenz, Y., M. Zarazaga, M. Lantero, M. Gastanares, F. Bacuero, and C. Torres. 2000. Antibiotic resistance in *Campylobacter* strains isolated from animals, foods and human in Spain in 1997-1998. Antimicrobial agents and chemotherapy. 44(2):267-271.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and et al. 1985. Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230:1350-3354.

Schorr, D., H. Schmid, H. Rieder, A. Baumgartner, H. Vorkauf, and A. Burnens. 1994. Risk factors for *Campylobacter* enteritis in Switzerland. Zbl. Hyg. 196:327-337.

Segreti, J., T. D. Gootz, L. J. Goodman, G. W. Parkhurst, J. P. Quinn, B. A. Martin, and L. J. Trenholme. 1992. High-level quinolone resistant in clinical isolates of *Campylobacter jejuni*. The journal of infectious diseases. 165:667-670.

Shapiro, R., L. Kumar, P. Phillips-Howard, J. Wells, P. Adcock, J. Brooks, M. Ackers, J. Ochieng, E. Mintz, S. Wahlquist, P. Waiyaki, and L. Slutsker. 2001. Antimicrobial resistant bacterial diarrhea in rural western Kenya. The journal of infectious diseases. 183(1701-1704).

Sharma, V., E. Dean-Nystrom, and T. Casey. 1999. Semi-automated fluorogenic PCR assays (Taqman) for rapid detection of *Escherichia coli* O157:H7 and other shiga toxigenic *E.coli*. Molecular and cellular probes. 13:291-302.

Shih, D. Y. 2000. Isolation and identification of enteropathogenic *Campylobacter spp.* from chicken samples in Taipei. Journal of food protection. 63(1):304-308.

Simango, C., and G. Rukure. 1991. Potential sources of *Campylobacter* species in the home of farm workers in Zimbabwe. Journal of tropical medicine and hygiene. 94:388-392.

Skjerve, E., L. M. Rovik, and O. Olsvik. 1990. Detection of *Listeria monocytogenes* in foods using immunomagnetic separation. Applied and environmental microbiology. 56:3478-3481.

Smith, K. E., J. M. Besser, C. W. Hedberg, F. T. Leano, J. B. Bender, J. H. Wicklund, B. P. Johnson, K. A. Moore, and M. T. Osterholm. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. The new england journal of medicine. 340(20):1525-32.

Smith, R. D. 1995. Veterinary Clinical Epidemiology, 2 ed. CRC press, Ann Arbor.
Smith, S., D. Olukoya, A. Fox, and A. Coker. 1998. Ribosomal RNA gene restriction fragment diversity amongst Penner serotype of Campylobacter jejuni and Campylobacter coli. Z. Naturforsch. 53c(65-68).

Smith, S. I., T. I. Sansa, and A. O. Coker. 1999. Antibiotic susceptibility patterns and beta-lactamase production of animal and human isolates of *Campylobacter* in Lagos, Nigeria. Verlag der Zeitschrift fur naturforschung. 54c:583-586.

Stanley, K., J. Wallace, J. Currie, P. Diggle, and K. Jones. 1998. The seasonal variation of thermophilic *campylobacters* in beef cattle, dairy cattle and calves. Journal of applied microbiology. 85:472-480.

Steele, M., B. McNab, L. Fruhner, S. DeGrandis, D. Woodward, and J. A. Odumeru. 1998. Epidemiological typing of *Campylobacter* isolates from meat processing plants by pulse-field gel electrophoresis, fatty acid profile typing, serotyping, and biotyping. Applied and environmental microbiology. 64(7):2346-2349.

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

Steinhauserova, I., K. Fojtikova, and J. Klimes. 2001. The incidence and PCR detection of *Campylobacter upsaliensis* in dogs and cats. Letters in applied microbiology. 31:209-212.

Steinhauserova, I., K. Fojtikova, and J. Matiasovic. 2001. Subtyping of *Campylobacter spp.* strains and the incidence in piglets. Acta veterinaria Brno. 70:197-201.

Stern, N., S. Green, N. Thaker, D. Krout, and J. Chiu. 1984. Recovery of *Campylobacter jejuni* from fresh and frozen meat and poultry collected at slaughter. Journal of food protection. 47(5):372-374.

Stern, N., M. Hernandez, L. Blankenship, K. Deibel, S. Doores, M. Doyle, H. NG, M. Pierson, J. Sofos, W. Sveum, and D. Westhoff. 1985. Prevalence and distribution of *Campylobacter jejuni* and *Campylobacter coli* in retail meats. Journal of food protection. 48(7):595-599.

Stern, N., K. Hiett, N. Cox, G. Alfredsson, K. Kristinsson, and J. Line. 2000. Recent developments pertaining to *Campylobacter*. Irish journal of agricultural and food research. 39:183-187.

Stern, N. J., M. R. S. Clavero, J. S. Bailey, N. A. Cox, and M. C. Robach. 1995. *Campylobacter spp.* in broilers on the farm and after transport. Poultry science. 74:937-941.

Stokes, M.E., C.S.Davis, and G.G. Koch. 2000. Categorical data analysis using the SAS system. 2nd ed. SAS Institute, Cary, NC.

Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous *campylobacter* infection: A swedish case-control study. Epidemiology and infections. 125:269-275.

Talsma, E., W. G. Goettsch, H. L. J. Nieste, P. M. Schrijnemakers, and M. J. W. Sprenger. 1999. Resistance in *Campylobacter* species: increased resistance to fluoroquinolones and seasonal variation. Clinical infectious diseases. 29:845-848.

Tay, S. T., S. D. Puthucheary, S. Devi, and I. Kautner. 1995. Characterisation of *Campylobacters* from Malaysia. Singapore medical journal. 36:282-284.

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

Tenover, F., R. Arbeit, R. Goering, P. Mickelsen, B. Murray, D. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-filed gel electrophoresis: criteria for bacterial strain typing. Journal of clinical microbiology. 33(9):2233-2239.

Threlfall, E., L. Ward, J. Frost, and G. Willshaw. 2000. The emergence and spread of antibiotic resistance in foodborne bacteria. International journal of food microbiology. 62:1-5.

Thunberg, R. L., T. T. Tran, and M. O. Walderhaug. 2000. Detection of thermophilic *Campylobacter spp.* in blood-free enriched samples of inoculated foods by the polymerase chain reaction. Journal of food protection. 63(3):299-303.

Thwaites, R. T., and J. A. Frost. 1999. Drug resistance in *Campylobacter jejuni*, *C. coli* and *C. lari* isolated from humans in North West England and Wales, 1997. Journal of clinical pathology. 52:812-814.

Uyttendaele, M., P. D. Troy, and J. Debevere. 1999. Incidence of *Salmonella*, *Campylobacter* jejuni, *Campylobacter coli*, and *Listeria monocytogenes* in poultry

carcasses and different types of poultry products for sale on the Belgian retail market. Journal of food protection. 62(7):735-740.

Van de Giessen, A., S. I. Mazurier, W. Jacob-Reitsma, W. Jansen, P. Berkers, W. Ritmeester, and K. Wernars. 1992. Study on the Epidemiology and control of *Campylobacter jejuni* in poultry broiler flocks. Applied and environmental microbiology. 58(6):1913-1917.

Van de Giessen, A. W., B. P. M. Bloemberg, W. S. Ritmeester, and J. J. H. C. Tilburg. 1996. Epidemiological study on risk factors and risk reducing measures for *Campylobacter* infections in Dutch broiler flocks. Epidemiology and infection. 117:245-250.

Vandamme, P. 2000. Taxonomy of the family Campylobacteriaceae, p. 3-26. In I. Nachamkin and M. Blaser (ed.), Campylobacter, vol. 2. ASM press, Washington DC.

Vanniasinkam, T., J. A. Lanser, and M. D. Barton. 1999. PCR for the detection of *Campylobacter spp.* in clinical specimen. Letters in applied microbiology. 28(1):52-56.

Varavithya, W., K. Vathanophas, L. Bodhidata, P. Punyaratabandhu, R. Sangchai, S. Athipanyakom, C. Wasi, and P. Echeverria. 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. Journal of clinical microbiology. 28(11):2507-2510.

Vasallo, F. J., P. M. Rabadan, L. Alcala, J. M. G. Lechuz, M. R. Creixems, and E. Bouza. 1998. Failure of ciprofloxacin therapy for invasive nontyphoidal Salmonellosis. Clinical infectious diseases. 26:535-536.

Velazquez, J., A. Jimenez, B. Chomon, and T. Villa. 1995. Incidence and transmission of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli*. Journal of antimicrobial chemotherapy. 35:173-178.

Waegel, A., and I. Nachamkin. 1996. Detection and molecular typing of *Campylobacter jejuni* in fecal samples by polymerase chain reaction. Molecular and cellular probes. 10:75-80.

Wagenaar, J., M. Van Bergen, D. Newell, R. Grogono-Thomas, and B. Duim. 2001. Comparative study using amplified fragment length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. Journal of clinical microbiology. 39(6):2283-2286.

Walker, R. D., and C. Thornsberry. 1998. Decrease in antibiotic susceptibility or increase in resistance? Journal of antimicrobial chemotherapy. 41(1):1-4.

Waller, D., and S. Ogata. 2000. Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in food. Applied and environmental microbiology. 66(9):4115-4118.

Wang, Y., W. M. Huang, and D. E. Taylor. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutation. Antimicrobial agents and chemotherapy. 37(3):457-463.

Wasfy, M., B. Oyofo, J. David, T. Ismail, A. El-Gendy, Z. Mohran, Y. Sultan, and L. Peruski. 2000. Isolation and antibiotic susceptibility of Salmonella, Shigella and *Campylobacter* from acute enteric infections in Egypt. Journal of health and population nutrition. 18(1):33-38.

Wassenaar, T., and D. Newell. 2000. Genotyping of *Campylobacter spp.* Applied environmental microbiology. 66(1):1-9.

Wedderkopp, A., E. Rattenborg, and M. Madsen. 2000. National surveillance of *Campylobacter* in broilers at slaughter in Denmark in 1998. Avian diseases. 44:993-999.

Weijtens, M. J. B. M., J. Plas, P. G. H. Bijker, H. A. P. Urling, D. Koster, J. G. Logtestijn, and J. H. J. Veld. 1997. The transmission of *Campylobacter* in piggeries; an epidemiological study. Journal of applied microbiology. 83:693-698.

Weijtens, M. J. B. M., R. D. Reinders, H. A. P. Urling, and J. Plas. 1999. *Campylobacter* infections in fattenning pigs; excretion pattern and genetic diversity. Journal of applied microbiology. 86:63-70.

Wesley, I., S. Wells, K. Harmon, A. Green, L. Schroeder-Tucker, M. Glover, and I. Siddique. 2000. Fecal shedding of *Campylobacter* and *Arcobacter spp.* in dairy cattle. Applied and environmental microbiology. 66(5):1994-2000.

White, P. L., W. Schlosser, C. E. Benson, C. Maddox, and A. Hogue. 1997. Environmental survey by manual drag sampling for *Salmonella enteritidis* in chicken layer house. Journal of food protection. 60(10):1189-1193.

Whyte, P., J. Collins, K. McGill, C. Monahan, and H. O'Mahony. 2001. The effect of transportation stress on excretion rates of *Campylobacters* in market-age broiler. Poultry sciences. 80:817-820.

Whyte, P., C. JD, K. McGill, C. Monahan, and H. O'Mahony. 2001. Distribution and prevalence of airborne microorganisms in three commercial poultry processing plants. Journal of food protection. 64(3):388-391.

Willis, W. L., and C. Murray. 1996. *Campylobacter jejuni* seasonal recovery observations of retail market broilers. Poultry science. 76:314-317.

Wilson, D. L., S. R. Abner, T. C. Newman, L. S. Mansfield, and J. E. Linz. 2000. Identification of Ciprofloxacin-resistant *Campylobacter jejuni* by use of a *fluorogenic* PCR assay. Journal of clinical microbiology. 38(11):3971-3978.

Witte, W. 1998. Medical consequences of antibiotic use in agriculture. Science. 279:996-7.

Witte, W., H.Tschape, I. Klare and G. Werner. 2000. Antibiotic in animal feed. Acta veterinary scandinavica. 93 (supplement): 37-45.

Wretlind, B., A. Stromberg, L. Ostlund, E. Sjogren, and B. Kaijser. 1992. Rapid emergence of quinolone resistance in *Campylobacter jejuni* in patient treated with Norfloxacin. Scandinavian journal of infectious diseases. 24:685-686.

Wu, R., G. Liu, and S. She. 2000. Carriage status, isolation and cultivation of *Campylobacter jejuni* from human, domestic animals and poultry. Chinese journal of veterinary science and technology. 30(1):13-16.

Young, C., R. Harvey, R. Anderson, D. Nisbet, and L. Stanker. 2000. Enteric colonisation following natural exposure to *Campylobacter* in pigs. Research in veterinary science. 68(1):75-78.

Yu, L., J. Uknalis, and S. Tu. 2001. Immunomagnetic separation methods for the isolation of *Campylobacter jejuni* from ground poultry meats. Journal of immunological methods. 256:11-18.

Zeger, S.L., and K.Y.Liang. 1986. Longitudinal data analysis for discrete and continuous outcomes. Biometrics. 42: 121-130.

Zirnstein, G., Y. Li, B. Swaminathan, and F. Angulo. 1999. Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyrA* resistance mutation by mismatch amplification mutation assay PCR and DNA sequence analysis. Journal of clinical microbiology. 37(10):3276-3280.