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THE DEVELOPMENT OF A STANDARDIZED IN VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING METHOD FOR *CAMPYLOBACTER* SPECIES

presented by

ROBERT ANDREW HANSON

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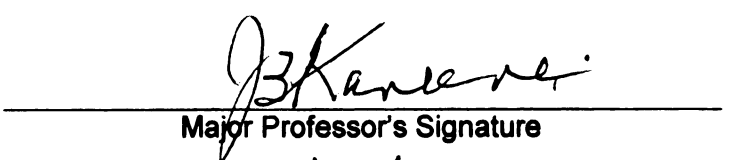

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THE DEVELOPMENT OF A STANDARDIZED IN VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING METHOD FOR *CAMPYLOBACTER* SPECIES

By

Robert Andrew Hanson

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ABSTRACT

THE DEVELOPMENT OF A STANDARDIZED IN VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING METHOD FOR *CAMPYLOBACTER* SPECIES

By

Robert Andrew Hanson

Campylobacter jejuni is the most commonly isolated sporadic foodborne pathogen in the United States. Efforts to monitor the prevalence and antimicrobial resistance patterns of *Campylobacter jejuni* require the development of a standardized in vitro antimicrobial susceptibility testing method to ensure accurate, reproducible and reliable reporting data. There are currently several published antimicrobial susceptibility testing methods for *Campylobacters*; however, there is no National Committee for Clinical Laboratory Standards (NCCLS) method available.

In order to develop a standardized method for the antimicrobial susceptibility testing of *Campylobacter spp.* three specific objectives were accomplished. First, define the optimal environmental growth conditions on artificial media allowing robust growth of *Campylobacter* isolates. Second, identify a Quality Control (QC) organism which would survive several passages on artificial media and exhibit a reproducible in vitro antimicrobial susceptibility profile. And third, define a testing method with preliminary QC ranges for each antimicrobial evaluated. Mueller-Hinton agar with 5% defibrinated sheep's blood provided optimal growth in a 10% CO_2 atmosphere at 42°C . ATCC 33560 *Campylobacter jejuni* produced the most reliable and reproducible MIC data points using the broth microdiluton method and has been endorsed by the NCCLS as a QC organism.

To my parents, Ron and Kim Hanson
thank you for all the love and support throughout the years

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" If you can fix the problem, then fix the problem and don't worry about
If you can't fix the problem, then don't worry about it"

Robert D. Walker
July 18, 1998

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INTRODUCTION

During the latter part of the 20th century, *Campylobacter jejuni* has become recognized as the most common isolated sporadic foodborne pathogen and the leading cause of bacterial gastroenteritis reported in the United States and has achieved worldwide attention among the international medical communities(1,2). *Campylobacter* organisms have long been recognized as a cause of diarrhea in cattle and sheep, but have only been recognized as an important cause of human illness for the last 25 years (2). *Campylobacter* was thought to be an opportunistic human pathogen when it was isolated from human blood cultures in the 1950s (3).

The most commonly identified species, *Campylobacter jejuni,* was first isolated from human stools in 1972 by a filtration technique developed for veterinary research (4). *Campylobacter jejuni* is commonly found as commensal of the gastrointestinal tract of wild and domesticated cattle, sheep, swine, goats, dogs, cats, and most varieties of fowl. Some of these animals, including cattle, pigs and poultry, are part of the human food chain (5). *C. jejuni* is not considered to be part of the normal intestinal flora in humans, thus when ingested via contaminated animal food products it can cause severe gastroenteritis. Meats originating from infected animals frequently become contaminated with intestinal contents during the slaughtering process.

The consumption of contaminated poultry is estimated to be responsible for 50 to 70 percent of sporadic bacterial gastroenteritis infections within the United States. It has been estimated that 70 to 90 percent of poultry products in the United States destined for human consumption are contaminated with *Campylobacter jejuni* (6). Other vehicles of infection include raw clams, unpasteurized goat's milk and cheeses and contaminated vegetables. Investigations of more than 50 outbreaks have also indicated that unpasteurized cow's milk can lead to bacterial enteritis by *Campylobacter jejuni* (6).

In 1997, laboratory -confirmed human bacterial gastroenteritis cases were made up as follows: 46% were campylobacteriosis, 26% were salmonellosis, 15% were shigellosis, 4.0% were E. coli O157:H7 infections, 1.6% were yersiniosis, and approximately 1% each were listeriosis, and vibrio infections. The aforementioned laboratory data was reported by the Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA), and the Food Safety and Inspection Service(FSIS) Food-Borne Disease Active Surveillance Network (FoodNet)(7). Data provided by the 1996 FoodNet surveillance program established *Campylobacter* as the most commonly isolated sporadic foodborne pathogen in the United States (1) and this trend continued in 1997 and 1998 (7,8)

Today, *Campylobacters*, particularly, *C. jejuni*, are known to be a leading cause of human gastroenteritis worldwide(1,7,8). *Campylobacter jejuni* infections occur throughout the year in the United States and other developed countries, showing sharp peaks in the summer and early fall.

As a result of incomplete national surveillance, the actual incidence of *Campylobacter* infections in the U.S. is not known. However, Passive reporting by laboratories across the country have indicated that *C.jejuni* is usually the more commonly isolated from fecal specimens provided by patients with diarrheal syndromes than *Salmonella* or *Shigella* (6).

The effect of campylobacteriosis is usually self-limiting in humans; however, in some cases re-infection may occur. It is estimated that annually there are between two and eight million cases of enteric campylobacteriosis in the U.S. with approximately 200-1000 deaths (9). The current treatment for campylobacteriosis includes re-hydration therapy with isotonic saline and antimicrobial agents, such as the flouroquinolones (10). Chronic sequelae of *C. jejuni* includes reactive arthritis, hemolytic uremic syndrome, and other gastrointestinal disorders.

One of the most important sequelae associated with *Campylobacter jejuni* is Guillian-Barre syndrome. Guillian-Barre syndrome is a subacute, acquired, demyelinating neuro-perpherial disorder. This condition is rare in the United States, but may be fatal to those suffering from the disease. Severe damage to the perpherial nervous system can result if the patient is left untreated (11). It is estimated that there are 5,000 cases of Guillian-Barre syndrome each year in the United States. Studies indicate that 36-40% of these cases follow a *Campylobacter* infection (12).

Disease Prevalence

In the United States, an estimated 2.1 to 2.4 million cases of human campylobacteriosis occur each year(2). Common symptoms that patients experience with laboratory-confirmed infections include fever, diarrhea, bloody stools and abdominal cramping(13). Bacteremia, septic arthritis, and extraintestinal symptoms are far less common (14).

The incidence of campylobacteriosis in HIV positive patients is much higher than in the general population. For instance, in Los Angeles County between 1983 and 1987, the reported incidence of campylobacteriosis in patients living with AIDS was approximately 519 cases per 100,000 population, which was 39 times higher than the rate in the general population (15). Recurrent infections and infection with antimicrobial resistant strains of *C. jejuni* are major complications faced by patients living with AIDS (16).

In the United States, infants have the highest age-specific *Campylobacter* isolation rate, approximately 14 per 100,000 person years. In young adolescents the isolation rates decline to approximately 4 per 100,000 person years. The peak isolation rate in neonates and infants has been attributed in part to susceptibility upon first exposure and to the low threshold for seeking medical care for infants. An interesting feature of the epidemiology of human campylobacteriosis is the high isolation rate among young adults, approximately 8 per 100,000 person years. The high rate of infection during early adulthood, which is pronounced in men, is thought to reflect poor food-handling practices and lack of proper cooking times, especially when cooking poultry. Among middle-aged and older adults, the isolation rate is < 3 per 100,000 person years (2).

Animal Reservoirs

Campylobacter jejuni is commonly found as commensal of the gastrointestinal tract of domesticated beef cattle, poultry, sheep, swine, goats, dogs, cats and rodents.

Animals and animal products have been identified as sources of infection in several outbreaks, and many of the *Campylobacter* serotypes that cause disease in humans have been isolated from animals.

C.jejuni is not considered to be part of the normal intestinal flora in humans, thus when ingested via contaminated animal food products it can cause severe gastroenteritis (17). *C.jejuni* appears to be normal commensal of all classes of bovines. Carcasses may become contaminated with intestinal contents during slaughter (18). One study conducted in Sweden showed the presence of *C. jejuni* in the feces of 17 out of 90 (19%) cattle, and another study conducted in Finland showed a *C. jejuni* incidence of 5.5% from fecal samples taken from 200 cattle (46).

A community outbreak of gastroenteritis in Vermont was traced to the consumption of unpasteurized milk produced at a commercial dairy farm. Two different testing methods showed a *C. jejuni* isolate from a sick patient and an isolate from a diseased cow to be the same serotype (47). Carcass contamination by *Campylobacter* species is more common in swine carcasses than in sheep, cattle, or goats. The danger of intestinal spillage is greater with the procedures used in dressing swine carcasses than with those used in dressing the carcasses ruminants (48).

Campylobacter species are frequently found in the intestinal flora of commercially raised birds and such wild birds as pigeons, crows, ravens, and seagulls (49). A Dutch study of broiler chickens found the highest rate of *Campylobacter* contamination from the months of June to September and the lowest in March.

Potential routes of entry of organisms into a flock include infection of newborn chicks from older birds, contamination of feed and water, and wild or game birds. Infection of poultry is often without clinical symptoms (50). Reservoirs in the poultry environment include beetles (51), unchlorinated drinking water (32), and farm workers (29,30,31). Vertical transmission (i.e. from breeder flocks to progeny), as seen in Salmonella species, has been hypothesized, but not widely accepted.

Because of its association with animals and their feces, it has been found in surface water and parts of the human food chain, resulting in its being classified as a zoonotic foodborne pathogen (52)

Transmission: Human to Human

Fecal-oral person-to-person infection has been documented for *C. jejuni*. As with other enteric pathogens, those in contact with excreta of infected people are at risk.(19). Human-to-human transmission has been reported from food handlers that are infected and carry the organism (20).

A food handler was the most probable source of an outbreak of acute gastroenteritis due to *Campylobacter jejuni* that occurred at a military base in Israel. Stool cultures were taken from 17 clinically affected as well as 23 asymptomatic soldiers.

In 6 of the 17 patients with enteritis (35%), *Campylobacter jejuni* serotype (ii) was isolated, while the stool cultures of all the asymptomatic soldiers were negative. The food handler had suffered from acute gastroenteritis before the outbreak but had not reported the illness. He was found to harbor the same serotype as the affected patients (21).

Transmission: Animal to Human

A majority of human *Campylobacter* infections are sporadic in nature. Sporadic *Campylobacter* infections usually occur from June to early August. A number of case-control studies have identified one of the most important risk factors associated with sporadic campylobacteriosis, specifically, handling raw and ingesting undercooked poultry, which eventually caused campylobacteriosis in humans.

Outbreaks usually occur during the spring and fall months in the U.S. Another outbreak vehicle, the consumption of unpasteurized milk, has been responsible for 30 of the 80 reported outbreaks of campylobacteriosis to the CDC between 1973 and 1992. Outbreaks caused by drinking unpasteurized milk often involve visits to farms (e.g., school field trips) during the temperate seasons (22).

Less frequent risk factors associated with campylobacteriosis include drinking milk from bird-pecked bottles, handling young companion pets like dogs and cats, especially pets with chronic and persistent diarrhea. Reports of serotype overlaps exists between clinical *C. jejuni* isolates recovered from humans, poultry, cattle, and swine indicating that foods of animal origin can contribute to a major pathway in the transmission of *C. jejuni* to humans(23).

Campylobacters and the Food Supply

Epidemiological evidence supports the hypothesis that raw agricultural products such as poultry, beef, raw milk are sources of human *Campylobacter* infections. Most chickens destined for human consumption is contaminated with *C. jejuni*, one study reported an isolation rate of 98% for retail chickens. The bacterial counts on the carcasses can often exceed 10^3 per 100 grams.

Skin and giblets have particularly high levels of contamination(24). In another study, raw milk samples from dairy farms in Tennessee yielded a contamination rate of 12% and all the isolates were identified as *C. jejuni(25).* Raw milk is thought to be contaminated with bovine feces; however, direct contamination of milk can also occur as a result of mastitis(26). *Campylobacters* can also be found in beef and other red meats. In one study, *C. jejuni* was recovered in approximately 5% of raw ground beef and in 40% of veal samples(27).

The Farm Environment

The control of *Campylobacter* contamination of farms has been a major topic of discussion between farm owners and the Food and Drug Administration. The goal remains to reduce the risk of contamination of food animal carcasses, such as poultry and beef products bound to enter retail venues(28). Epidemiological studies indicate that strict hygiene reduces intestinal carriage in food-producing animals(29,30,31). In field studies, poultry flocks that drank chlorinated water had lower intestinal colonization rates than poultry that drank unchlorinated water(30,32).

Meats originating from infected animals frequently become contaminated with intestinal contents during the slaughtering process. Bacterial counts on carcasses usually increase during the slaughtering process 13). In one study, up to a 1000 fold increase in bacterial counts on carcasses was reported during transportation to slaughter(33).

In another study, the defeathering and evisceration of chickens(34) and turkeys at slaughter increased the bacterial counts by approximately 10 to 100 fold during those processes (35).

Treatment of Campylobacteriosis

The primary treatment for most campylobacteriosis patients include fluid and electrolyte replacement. Severely dehydrated patients should receive rapid intravenous infusion of normal saline (0.9%) which will expand the patients blood volume(36). *Campylobacter* infections are usually self limiting, antibiotic therapy may be indicated for patients who have a high fever, bloody diarrhea with more that 8 stools in a 24 hour period, immunocompromised patients suffering from HIV or chemotherapy, and patients with bacteremia or septicemia (36).

The enteric disease caused by *C. jejuni* has responded to several different antimicrobial agents, including the flouroquinolones. Unfortunately, the enteric disease caused by this organism may be clinically indistinguishable from those caused by other intestinal pathogens of humans, such as *Salmonella*, *Shigella* and *Vibrio cholera*.

The recommended antimicrobial therapy for gastroenteritis caused by these organisms is ciprofloxacin, a flouroquinolone (10); however, the plasma concentration of ciprofloxacin required to inhibit the growth of *C. jejuni* may be higher than the concentration required to inhibit growth of *Salmonella*, *Shigella* and *Vibrio cholera* which may enhance the selection for resistant isolates of *C. jejuni*.(37).

Antimicrobial Resistance

Prior to 1989, there were no reported incidences of drug resistance to fluoroquinolones from any laboratory isolated and confirmed *Campylobacter* organisms. Since 1989, fluoroquinolone resistant *Campylobacter* isolates have been reported by several European countries and more recently, in the United States (38). The incidence of fluoroquinolone-resistant *C. jejuni* in Europe has been associated with an increase in the use of fluoroquinolones in human and veterinary medicine.(10,38).

The lack of prudent use of antimicrobial agents, namely flouroquinolones, in both human and veterinary medicine may contribute the current problem of antimicrobial resistance among enteric pathogens(39). The rate of antimicrobial resistant enteric infections is highest in the developing world, where the use of antimicrobial drugs in humans and animals is relatively unrestricted. A study conducted in 1994 reported that most of the clinical isolates of *C. jejuni* from U.S. army troops in Bangkok, Thailand were resistant to ciprofloxacin(40).

The first fluoroquinolone approved in the United States for use in human medicine was norfloxacin in 1986, and shortly thereafter in 1988, ciprofloxacin was approved for use in human medicine. Since 1988, there has been several other fluoroquinolones approved in the U.S. for use in human medicine such as levofloxacin, ofloxacin, lomofloxacin, fleroxacin and trovafloxacin. Enrofloxacin was approved for use in dogs and cats in 1989. Currently, enrofloxacin is being used to treat dogs, cats, chickens, turkeys, and beef cattle. Furthermore, difloxacin, orbifloxacin, and marbofloxacin are being used to treat dogs and/or cats.

Sarafloxacin, which is no longer available, was used on poultry farming units to promote better health and growth. After flouroquinolone use in poultry was approved in Europe, resistant *C. jejuni* strains emerged rapidly in humans during the early 1990s. Such widespread use of fluoroquinolones in human and veterinary medicine within the United States could result in an increased incidence of antimicrobial drug resistance similar to the incidence rates of antimicrobial drug resistance evident in some European countries(39).

Concern for the development of bacterial resistance to the fluoroquinolones resulted in a meeting between the Infectious Disease Society of America (IDSA), the Centers for Disease Control and Prevention (CDC), and both the human and the veterinary components of the Food and Drug Administration's (FDA), specifically the Center for Drug Evaluation and Research (CDER) and the Center for Veterinary Medicine (CVM), respectively.

A recommendation that resulted from this meeting was that when a pharmaceutical company has a fluoroquinolone approved for use in food animal species, the company is responsible for establishing a nationwide resistance monitoring system to monitor the possible variation in susceptibility patterns of the target pathogen in relation to time. Since this recommendation, enrofloxacin, sarafloxacin, orbifloxacin, difloxacin and marbofloxacin have been approved for use in veterinary medicine with the approval of other fluoroquinolones pending. The USDA/CDC/FDA National Antimicrobial Resistance Monitoring System was established to monitor drug resistance in *Salmonella* isolates from animals and humans. Antimicrobial testing of *Campylobacter spp.* was added to the program in 1998.

In 1988 the Food Safety and Inspection Service (FSIS), a department of the United States Department of Agriculture (USDA), initiated a scientific collaboration with the Agriculture Research Service (ARS) to strengthen and establish an official interrelationship between the two agencies to cooperate indefinitely on food safety research efforts(41). In 1995 an important and unique public health initiative was established known as the CDC/FDA/FSIS Foodborne Diseases Active Network called FOODNET (1,7,8).

The justification for the Development of an In Vitro Susceptibility Testing Method for *Campylobacter* spp.

In order to address and implement the recommendations for drug resistance monitoring set forth by the aforementioned agencies, the development of a standardized in vitro antimicrobial susceptibility testing method was necessary to ensure accurate, reproducible and reliable in vitro antimicrobial susceptibility testing of *Campylobacter spp.*

The National Committee for Clinical Laboratory Standards (NCCLS) has defined the criteria for developing such a standard in their M23-T3 and M37-A documents. For the in vitro antimicrobial susceptibility testing of almost all other bacterial pathogens, laboratories doing such tests follow the procedures recommended by the NCCLS.

Tests results that are generated without NCCLS standardization may not be reproducible and thus, may not be verifiable. While there are several published procedures for the in vitro antimicrobial susceptibility testing of *C. jejuni* (42,43,44,45), there is no NCCLS approved standards for performing these tests.

The NCCLS testing procedure required the standardization of a quality control organism, including defining the Minimal Inhibitory Concentration (MIC) ranges for each antimicrobial agent tested, defining the artificial media required for optimal bacterial growth, optimal atmospheric conditions and interpretive criteria for antimicrobial susceptibility ranges.

The selection of quality control organism is based on its phenotypic stability as determined by its colony characteristics, the survival of the organism following numerous passages on artificial media, and genotypic stability as determined by its reproducible in vitro antimicrobial susceptibility profile. In order for *C. jejuni* to be included in a national resistance monitoring system, as recommended by the aforementioned agencies, a standardized method for performing in vitro antimicrobial susceptibility testing on clinical *Campylobacter* isolates was required.

Testing Objectives

There were three specific objectives to accomplish in order to develop a standardized method for the in vitro antimicrobial susceptibility testing of *Campylobacter* species.

First, define the optimal growth conditions on artificial media capable of producing luxuriant growth of *Campylobacter* isolates recovered from biological and/or environmental sources (i.e. feces, blood, carcasses, water).

Second, identify a Quality Control (QC) organism that possess genotypic and phenotypic stability based on the QC organism's capacity to survive numerous passages on artificial media and the reproducibility of the QC organism's in vitro antimicrobial susceptibility profile. And third, define a testing method and define the preliminary QC ranges for each antimicrobial agent evaluated.

LITERATURE REVIEW

Published, non standardized antimicrobial testing methods for *Campylobacter jejuni*

A study conducted by Huang et al., utilized heart infusion agar media supplemented with 5% rabbit blood (HIAB) to recover frozen *Campylobacter* isolates and then incubated them for 48 h at 35°C, and subsequently restreaked the recovered isolates onto a fresh HIAB plate and incubated the isolates for an additional 24 h at 42°C to capture the thermophilic species. The suspensions of the organisms used for the broth microdilution assay were prepared in 5 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and adjusted to equal the turbidity of a 0.5 McFarland standard (1 x 108 CFU/ml). When performing antimicrobial susceptibility testing using the broth microdilution method the Huang Group used the guidelines established by the NCCLS Document M7-A2 for bacteria that grow aerobically.

A study conducted by Aarestrup et al., used a selective enrichment phase with Preston broth from isolates collected from animal fecal samples. After the Preston broth was inocculated, the suspension was incubated for 18 to 24 h at 42°C, to enhance the growth of thermophilic *Campylobacter spp.*, in a microaerobic atmosphere (approximately 6% O2, 7% CO2, 7% H2, and 80% N2) created by a gas generation system. One loop of the broth was then transferred to mCCDA (Oxoid CM739 plus selective supplement SR 155E) selective media plate for performing a Tenover et al. modified agar dilution method.

A study conducted by Van Looveren et al., used different minced farm animal meats to recover *Campylobacter* organisms. The meat samples were collected with a brucella broth (Oxoid, Basingstoke, UK) moistened sterile cotton swab. In the lab the swabs were homogenized into Preston selective broth (Oxoid) and incubated in a micro-aerophilic atmosphere at 42°C for 48 h. The enrichment suspension was then streaked on to modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) and incubated at 42°C for 24-120 h. The susceptibility testing was conducted using the agar dilution method based on guidelines established by the NCCLS Document M7-A4.

A study conducted by Gaudreau et al., used *Campylobacter* isolates recovered from human samples. Upon collection the samples were frozen in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md) supplemented with 15% (v/v) glycerol. Inocula suspension was prepared in Mueller-Hinton broth (BBL Microbiology Systems) at a density adjusted to a 0.5 McFarland turbidity standard for the disc diffusion and diluted 1:10 for the agar dilution. Both testing methods used the same inoculum suspension to reduce intra-laboratory variation. The inoculated plates were incubated at 35°C under a microaerophilic atmosphere obtained with a glass generator envelope (Difco), for 48 h. Susceptibility testing criteria for the agar dilution and the drug concentrations in the discs were those of the NCCLS.

A study conducted by Huysmans et al., used 100 strains of thermophilic *Campylobacters* isolated from stools of patients suffering from acute diarrhea. The MICs of the 100 clinical isolates were determined by agar dilution using methods similar to those recommended by the NCCLS Document M7-A3, using Mueller-Hinton agar containing 5% lysed horse blood.

Zones diameters were determined on the same medium using NCCLS strength antimicrobial discs and similar methodology. All results were read after 48 h incubation at 37°C in a microaerophilic atmosphere (5% O2, 10 CO2, 10% H2, and 75% N2).

Agar Dilution and broth microdilution remain the standard methods of determining the susceptibility of *Campylobacters* to antimicrobial agents. However, this review of literature reveals that there is no consensus about the optimal medium, the requirement of blood supplementation, the temperature of incubation, or the time of incubation. Temperature and time of incubation , for instance, vary from 42°C for 24 h to 37°C for 48 h. For agar dilution testing, Mueller-Hinton agar containing 5% sheep blood, incubated in anaerobic jars with a gas mixture containing 5% O2, 10% CO2 and 85% N2 for 48 h at 37°C, is the method most frequently used at the Centers for Disease Control and Prevention in the United States, whereas cation-adjusted Mueller-Hinton broth containing 5% lysed horse blood, incubated as described above, is used by the CDC for broth microdilution assays. Other methods will produce acceptable results, but it is critical that quality control strains be tested in parallel to ensure the accuracy of the method.

The choice of method depends on several factors, including cost, ease of performance, personal preference and experience and the availability of methods in each laboratory. Susceptibility tests for *Campylobacter* are not yet standardized, and consequently the literature contains some variability in susceptibility data reported. For the interpretation of the results, breakpoints recommended by the NCCLS for aerobic bacteria have been used in most cases. However, national and international breakpoints or breakpoints established through population distribution studies have also been utilized.

MATERIAL AND METHODS: GENERAL

Materials

Bacterial Strains

A total of 71 *Campylobacter* isolates collected from human stool cultures and farming animals have been screened for use as a potential QC organism. Four of these isolates were submitted by Dr. Frank Aarestrup from the Danish Veterinary Laboratory in Denmark. Four were submitted by Dr. Laura Piddock from the University of Birmingham, United Kingdom. Six isolates were submitted by Dr.Konkel from the University of Washington and the remaining isolates were submitted by Dr. Walker from Michigan State University, College of Veterinary Medicine, who is currently at the CVM-FDA. Three type strains from the American Type Culture Collection (ATCC) were also included in the study: *C. jejuni* ATCC 33560, *C. jejuni* ATCC 43430, and *C. jejuni* 43470.

Of the 71 isolates, 21 were identified as *Campylobacter jejuni.* Of these 21 isolates, three isolates, *C. jejuni* ATCC 33560, *C. jejuni* E97-2805 from Dr. Konkel's laboratory and *C. jejuni* 4239-928 from Dr. Walker's laboratory were selected for subsequent testing in five laboratories. The five participating laboratories included the Clinical Microbiology Institute (CMI) in Wilsonville, OR., MRL: Pharmaceutical Services (MRL) in Herndon, VA., USDA-ARS (USDA) in Athens, GA., Duke University Medical Center (Duke) in Durham, NC., and Michigan State University (MSU) in East Lansing, MI.

To enhance and better visualize the cell walls of the *Campylobacter* isolates a modified Gram staining method was employed using carbofusion stain instead of the standard saffarin counterstain. Hippurate hydrolysis and oxidase testing was also performed to identify the isolates. Criteria for subsequent testing included survival of numerous passages on artificial media, growth characteristics, and susceptibility profile. One isolate, 4239-928 died after being shipped to participating laboratories and was not tested.

Antimicrobial agents

Eight different antimicrobial agents were used in this preliminary study. These included, amoxicillin, gentamicin, doxycycline, ciprofloxacin, tetracycline, erythromycin, trimethoprim/sulfamethoxazole and nalidixic acid for the broth microdilution method. All the antimicrobials were supplied by pharmaceutical companies as standard powders with known potencies. The compounds were prepared according to the recommendations by the manufacturers and NCCLS guidelines.

Antimicrobial disks

Zones of inhibition were determined using commercially available disks. The concentrations of the antimicrobial disks were; trimethoprim/sulfamethoxazole 15ug, erythromycin 15μg, nalidixic acid 30μg, ampicillin 10μg, gentamicin 10μg, ciprofloxacin 5μg, doxycycline 30μg, and tetracycline 30μg.

Two lots of disks were used for each antimicrobial agent. The disks were supplied by Becton Dickinson and shipped by Michigan State University to the participating laboratories.

Environmental Materials

Campy-Gas Generation Pouches (BBL) and a CO_2 incubator

Growth characteristics and antimicrobial MIC profiles of clinical *Campylobacter* isolates were investigated using commercially available Campy-pouches, which provides a microaerophilic environment, and a CO_2 incubator set at 10% concentrations . The preliminary disk diffusion and broth microdilution testing that was conducted using a CO_2 incubator was set to deliver a 10% CO_2 concentration. Based on a decision by the National Committee for Clinical Laboratory Standards-Veterinary Antimicrobial Susceptibility Testing (NCCLS-VAST) subcommittee, any subsequent *Campylobacter* antimicrobial susceptibility testing will be conducted using incubator set to deliver a 5% CO_2 concentration.

Zip-loc bag system

Growth characteristics and antimicrobial MIC profiles of clinical *Campylobacter* isolates were also investigated using commercially acquired plastic Zip-loc bags filled with a gas mixture (85% N_2, 5% O_2, and 10%CO_2). It was difficult to standardize the amount of gas entering the bags and that may contribute a source of error and lack of accuracy and precision to the testing methods.

Methods: Preparation of *Campylobacter* suspensions

A standard stock suspension of *C. jejuni* was prepared, using cation adjusted Mueller-Hinton broth, to achieve a final concentration of 0.5 McFarland, which is approximately 10^8 colony forming units.

When applicable, the broth microdilution and disk diffusion testing methods used the same standard stock suspension in order to minimize inoculum variations and other sources of error that may result from preparing two separate standard stock suspensions.

Disk Diffusion

Disk diffusion testing was performed as described in the NCCLS document M2-A6. Mueller-Hinton blood agar plates (150mm), supplemented with 5% sheep's blood. Inocula were prepared using cation adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) to match an optical density equal with a 0.5 McFarland Standard. A sterile cotton swab was used to inoculate the 150 mm blood agar plates by rotating the plates three consecutive times, 60 degrees each time, to ensure a uniform distribution of inocula across the surface of the plates.. The disk diffusion plates were incubated at 37°C in a 10% CO_2 atmosphere for 48 hours prior to measuring the zone of inhibition diameters.

Broth Microdilution

Broth microdilution MIC testing was performed according to NCCLS document M7-4A. During preliminary testing both Brucella and Mueller-Hinton broth was utilized and the growth patterns of the *Campylobacter* isolates were very reproducible and there seemed to be no microbiological growth advantage of either broths. A decision was made to use Cation adjusted Mueller-Hinton broth because it was more cost effective and more readily available. The inocula was prepared by suspending the *Campylobacter* isolates in cation adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) to achieve a turbidity of 0.5 McFarland standard.

The suspension was further diluted with sterile water to provide a final inoculum density of 5.0 x 10^5 CFU/ml in the wells of the broth microdilution trays. Colony counts were preformed on each inocula to ensure appropriate inocula concentrations. Following the inoculation of the antimicrobial trays, the trays were incubated at 37°C for 48 hours in a 5% and 10% CO_2 environments.

METHODS AND MATERIALS: DETAILED

Objective one: Define the optimal growth conditions, on artificial media, capable of producing luxuriant growth of *Campylobacter* isolates recovered from biological and/or environmental sources (i.e. feces, blood, carcasses, water)

Media

Various media formulations were investigated to determine the optimal and most reliable artificial growth media for in-vitro cultivation of clinical and laboratory attenuated strains of *Campylobacter.*

Both agar and broth media was investigated. The criteria for the selection of a suitable growth and antimicrobial susceptibility testing medium was based on its ability to provide optimal growth of *Campylobacter* isolates under varying atmospheric conditions. The mediums tested included cation-adjusted Mueller-Hinton broth, Brucella broth, Mueller Hinton agar, and Mueller-Hinton agar supplemented with 5% defibrinated sheep blood.

Temperature

Most *Campylobacter* organisms are thermophilic and thrive within a temperature range of 37°C to 42°C., these two temperature ranges were investigated in this study.

Incubation Period

The incubation period is directly correlates with the Incubation temperature. The incubation times tested were 24 and 48 hours.

Moisture content of the Incubator or Campy-Gas Generation Systems

The clinical *Campylobacter* isolates that were cultivated in a moist environment flourished and exhibited watery colony characteristics as compared with isolates that were cultivated within an environment lacking moisture. An incubator with a water pan was used to achieve a moisture-rich environment, along with moisture producing Campy-pouches and jars for isolate cultivation and testing.

Objective two: Identify a Quality Control (QC) organism that possess genotypic and phenotypic stability based on the QC organism's capacity to survive numerous passages on artificial media, and the reproducibility of the QC organism's in vitro antimicrobial susceptibility profile.

The second objective involved the identification of a quality control isolate that could represent the *Campylobacter* genus for in vitro antimicrobial susceptibility testing purposes. By using broth microdilution testing method, the MIC profiles of several *Campylobacter* isolates were obtained and evaluated based on genetic and phenotypic stability and growth survival characteristics on numerous passages on artificial media.

Initially 71 Campylobacter isolates were under investigation, these included 3 ATCC strain and 68 clinical isolates. Of the original 71 strains tested, 3 strains were selected for interlaboratory testing. The criteria for selecting the 3 strains were based on their stable growth patterns over numerous passages on artificial media and consistent and reproducible MIC data values.

Objective three: Define the preliminary Quality Control ranges for the in-vitro antimicrobial susceptibility testing of 8 antimicrobial agents against the selected QC organisms.

The third objective involved testing 3 potential quality control *Campylobacter* isolates in 5 laboratories. The 5 participating laboratories performed disk diffusion and broth microdilution testing methods on 3 potential quality control isolates. The broth microdilution trays and the disk diffusion plates were placed in an incubator set at 37°C in 10% CO_2 for 48 hours.

Based on the reproducibility of the broth microdilution MIC profiles and disk diffusion zone diameters reported by all 5 laboratories, one isolate was chosen to be tested in the final phase of the study and the dilution range relative to the MICs of the clinical isolates. During preliminary testing at Michigan State University, one manufacturer of MH agar (BBL) was used for making disk diffusion plates and one manufacturer of MH broth (Difco) was used for preparing broth microdilution trays. The Mueller-Hinton broth (Difco) used in the antimicrobial dilution trays were prepared by PML in Portland, Oregon.

RESULTS

Objective one

Media

There was no significant difference in the optical density readings after the incubation of *Campylobacter* suspensions for either Mueller-Hinton or Brucella broths under investigation. Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) has the advantage of being more widely available and is currently being used as the broth of choice for the in-vitro antimicrobial susceptibility testing of numerous other organisms and for those two reasons, Mueller-Hinton is the broth of choice for *Campylobacter* studies.

Optimal growth on agar surface studies were also conducted. *Campylobacter* isolates were streaked on MH agar and MH agar supplemented with fresh and old sheep's blood. The addition of sheep's blood resulted in a final Mueller-Hinton Blood (MHB) agar concentration of 5%. A non-selective Enriched Blood Agar (EBA) was also investigated. No growth was exhibited by all isolates tested using the non-supplemented MH agar.

Abundant growth was observed when testing the MHB agar and the EBA plates. The growth patterns between the MHB and EBA are indistinguishable and both provide for abundant growth of the organism; however, selection of MHB agar for future susceptibility testing of *Campylobacter* organisms is based on its wide distribution among laboratories and it has been commonly used for susceptibility testing of other clinical isolates.

Temperature

Campylobacter isolates will grow at 37°C and 42°C with optimal growth at 42°C occurring in 24 hours.

At a previous NCCLS-VAST subcommittee meeting, the working group selected 35-37°C for in vitro susceptibility testing since they felt time was not a factor and all laboratories have 37°C incubators. Based on that recommendation, any subsequent studies will be performed at 35-37°C.

Incubation period

The incubation period is related to the temperature of the testing environment. At 35-37°C the incubation period is 48 hours, whereas at 42°C the incubation period is 22-24 hours. An incubation period of 48 hours was used for all test conditions.

Moisture Content of the Incubator or Campy-Gas Generation systems

Placing water in the bottom of the incubators or using the Campy-bag or Campy-jar system will increase the humidity and produce abundant growth exhibiting watery *Campylobacter* colony characteristics. Optimal growth can be achieved by addition of water to an incubator or using Campy-pouches or Campy-jars which allows the organism to flourish.

Objective two

A total of 71 clinical *Campylobacter* isolates were tested for their ability survival numerous passages on artificial media and growth characteristics. Those isolates who died after only a few passages were deemed not suitable and would not represent a viable quality control organism. Of the 71 isolates observed, 21 isolates were selected for broth microdilution testing to narrow the field to 3 potential quality control organisms.

Most clinical isolates clustered around the QC ranges for each of the antimicrobial agents tested. Three isolates generated the most accurate and reproducible MIC data points among the 15 originally selected. The 3 selected for further testing in phase three were *Campylobacter jejuni* ATCC 33560, *Campylobacter jejuni* E97-2805 and *Campylobacter jejuni* 4239-928.

Objective three

Three potential quality control isolates were investigated. *Campylobacter jejuni* ATCC 33560, *Campylobacter jejuni* E97-2805, and Campylobacter jejuni 4239-928. *C. jejuni* 4239-928 died out shortly after the 3 isolates were shipped, consequently it was dropped from the study. For the two remaining candidates, the broth microdilution MIC data values reported exhibited accurate and reproducibly MIC data points among all 5 laboratories. Preliminary testing was done in five laboratories using the broth microdilution and the disk diffusion methods with eight antimicrobial agents.

Broth microdilution and disk diffusion data for isolate ATCC 33560 are shown in appendices A-D. Broth microdilution data values for isolate E97-2805 and disk diffusion data for ATCC 33560 and E97-2805 are not included in this thesis paper. While both isolates performed reasonably well against most drugs, there were some decrepencies.

The MIC ranges for isolate E97-2805 were off scale for both tetracycline and doxycycline. However, this isolate was slightly more active than isolate ATCC 33560 when tested against ciprofloxacin and nalidixic acid. Both isolates performed poorly when tested against trimethoprim/sulfamethoxazole (table 8), suggesting that this antimicrobial may not be an appropriate drug to test.

Overall, *C. jejuni* ATCC 33560 generated more reproducible data points with less variability between participating labs than *C. jejuni* E97-2805. When testing the ATCC 33560 isolate, using the broth microdilution method, all antimicrobial agents tested produced NCCLS acceptable MIC data values, which were between 2 to 3 dilutions away from the mean MIC values, except for Trimethoprim/Sulfamethoxazole.

The agents that performed very well overall include nalidixic Acid (Table 1), tetracycline (Table 2), gentamicin (Table 3), amoxicillin (Table 4), ciprofloxacin (Table 5), erythromycin (Table 6), doxycycline (Table 7). trimethoprim/sulfamethoxazole (Table 8) should not be used for antimicrobial susceptibility testing of organisms in this genus. Data collected from the disk diffusion testing exhibited zone diameters with excessive variability and poor reproducibility among the 5 laboratories. Preliminary testing was performed using the disk diffusion testing method with eight antimicrobial agents.

The resulting zones of inhibition varied considerably between the five laboratories and even within a laboratory. Interlaboratory variation was seen when ampicillin was tested against isolate E97-2805. Examples of intralaboratory variation with isolate ATCC 33560 may be seen with numerous isolate/drug combinations.

We hypothesized that this variation was due to the growth patterns on the plates and how the plates were read in relation to the light source. In other words, the zone of inhibition could appear as a hologram with the size of the zone of inhibition changing with respect to how the light reflected off the surface of the medium. To investigate the possible cause of this phenomena we looked at the effect variations in organism concentrations had on the size of the zone of inhibition.

We tested isolate 33560 at concentrations of 10^7, 10^8, 10^9 CFU/ml. There was no difference found in regards to the zone sizes. We also looked at the effect of varying concentrations of CO_2 had on the size of the zone of inhibition. Carbon dioxide concentrations of 5%, 8%, and 10% were investigated.

We found that this had no effect on the variation seen in the zone diameters. Disk diffusion should not be utilized for antimicrobial susceptibility testing of *Campylobacters* until precise growth characteristics can be identified that would eliminate variations in zone size and end point determinations.

DISCUSSION

The optimal environmental conditions, as endorsed by the NCCLS-VAST subcommittee, to support luxurious growth of *Campylobacter* organisms include using a Campy-Gas Generation System or a mechanical incubator with a CO_2 concentration setting of 10% along with a temperature setting of 37°C. The incubation period should be 48 hours. However, since there was only a slight difference in growth patterns between Mueller-Hinton broth tested at 5% and 10%, the NCCLS-VAST subcommittee recommended that 5% CO_2 be adopted as the standard CO_2 concentration since most clinical laboratories have access to a 5% CO_2 incubator.

The moisture content of the Campy-Gas Generation Systems allow the *Campylobacter* organisms to flourish and grow in abundance. The *Campylobacter* isolates grew better in an environment with a high moisture content when compared with the same environment lacking moisture. When using a mechanical incubator, water in a tray with a large surface area should be placed on the bottom of the incubator to enhance the growth of *Campylobacter* organisms. A Zip-loc bag system was investigated during phase one of the study.

A gas mixture containing 85% N_2, 5% O_2 and 10% CO_2 was injected into a commercially acquired zip-loc bag. The *Campylobacter* isolates exhibited good growth with this system; however, it was difficult to standardize the amount of gas that was injected into the bag and the added expense of a special gas cylinder proved to be unpractical as a standardized testing condition.

There are several different types of media that will support the growth of *Campylobacter* organisms; however, both Mueller-Hinton broth(Difco) and agar (BBL) preformed the best, supporting consistent and rapid growth of all clinical isolates tested.

Mueller-Hinton broth and agar are readily available and commonly used for the in-vitro antimicrobial susceptibility testing of numerous other bacteria species. One of the methods suitable for antimicrobial susceptibility testing of *the Campylobacter* genus is broth microdilution. This method generated reproducible and verifiable MIC data values. A recommendation to the NCCLS-VAST subcommittee was made to discontinue the use of disk diffusion for the antimicrobial susceptibility testing of *the Campylobacter* genus.

Based on data points collected and evaluated, disk diffusion is not a valid method of choice for antimicrobial susceptibility testing of the *Campylobacter* genus at this time, due to its excessive variability in zone diameter interpretations, which makes the interpretation of precise zone size difficult. Data tables for *Campylobacter jejuni* ATCC 33560 using the broth microdilution method can came be found in the appendices. Of all the antimicrobial agents tested in this study, only one agent, Trimethoprim/Sulfamethoxazole, is not recommended for susceptibility testing of the *Campylobacter* genus .

The overall performance of *Campylobacter jejuni* ATCC 33560 isolate during the broth microdilution testing method and concurrent data analysis has reinforced our decision to recommend that the *Campylobacter jejuni* ATCC 33560 isolate be used as a quality control isolate for the in vitro antimicrobial susceptibility of the *Campylobacter* genus.

SUMMARY and CONCLUSIONS

The recommended optimal environmental conditions for the antimicrobial susceptibility testing of *Campylobacter* species includes using a Campy-Gas Generation System or a 10% CO2 incubator set at 37C with an incubation period of 48 hours. *Campylobacter* isolates should be streaked onto Mueller-Hinton agar supplemented with 5% sheep's blood and Mueller-Hinton broth (Difco) should be used for inocula suspension preparation and antimicrobial susceptibility testing.

The broth microdilution method using Mueller-Hinton (Difco) broth produces reproducible and reliable antimicrobial susceptibility results. The disk diffusion method should not be used for the antimicrobial susceptibility testing of *Campylobacters* since this testing method produced excessive variability, poor reproducibility and inconsistent holographic zone sizes among the 5 laboratories, which makes interpertation challenging.

The QC organism for the antimicrobial susceptibility testing of the *Campylobacter* genus should be *Campylobacter jejuni* ATCC 33560. This specific isolate produced the most reproducible and reliable MIC data points among the 5 laboratories and has exhibited a robust growth characteristic throughout all testing objectives in this preliminary study.

CURRENT AND FUTURE RESEARCH

Based on the data collected from this preliminary broth microdilution study, the NCCLS-VAST subcommittee has endorsed the aforementioned environmental testing conditions for the *Campylobacter genus.* Meanwhile, testing of *C. jejuni* ATCC 33560 by the "gold standard" agar dilution method has been conducted in 10 laboratories worldwide. Based on the data collected from this agar dilution study of *C. jejuni* ATCC 33560, the NCCLS-VAST and AST has endorsed the agar dilution method as one of the standard antimicrobial susceptibility testing method for *the Campylobacter genus* and has also endorsed *C. jejuni* ATCC 33560 as the QC organism.

MIC data and antimicrobial breakpoints from the agar dilution study will be published in upcoming NCCLS M31 and M7 Documents. Further investigation of the broth microdilution testing method using 3 different manufacturers of Mueller-Hinton broth in 10 different laboratories worldwide will be conducted to validate and this testing method as a standard antimicrobial susceptibility testing method for the genus *Campylobacter.*

APPENDICES (A-D)

APPENDIX A

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16	10					10
8		3	2	5		10
4		7	8	5	8	28
2						
1						
0.5						
0.25						
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	16	5.2	4.8	6	4	7.33
Mode	16	4	4	4	4	4
Min	16	4	4	4	4	4
Max	16	8	8	8	4	16
Range	1	2	2	2	1	3

Table 1. BMD (Nalidixic Acid)

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4						
2				1		1
1	10	6		9		25
0.5		4	8		6	18
0.25			2		2	4
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	1	0.8	0.45	1.1	0.438	0.77
Mode	1	1	0.5	1	0.5	1
Min	1	0.5	0.25	1	0.25	0.25
Max	1	1	0.5	2	0.5	2
Range	1	2	2	2	2	4

Table 2. BMD (Tetracycline)

APPENDIX B

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4						
2	10	8	1	4		23
1		2	7	6	4	19
0.5			2		3	5
0.25					1	1
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	2	1.8	1	1.4	0.719	1.41
Mode	2	2	1	1	1	2
Min	2	1	0.5	1	0.25	0.25
Max	2	2	2	2	1	2
Range	1	2	3	2	3	4

Table 3. BMD (Gentamicin)

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4						
2	2			5		7
1	8	10	5	5	8	36
0.5			5			5
0.25						
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	1.2	1	0.75	1.5	1	1.1
Mode	1	1	1	1	1	1
Min	1	1	0.5	1	1	0.5
Max	2	1	1	2	1	2
Range	2	1	2	2	1	3

Table 4. BMD (Amoxicillin)

APPENDIX C

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4						
2						
1						
0.5	10					10
0.25		10	10	7	7	34
0.125				3	1	4
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	0.5	0.25	0.25	0.212	0.234	0.296
Mode	0.5	0.25	0.25	0.25	0.25	0.25
Min	0.5	0.25	0.25	0.125	0.125	0.125
Max	0.5	0.25	0.25	0.25	0.25	0.5
Range	1	1	1	2	2	3

Table 5. BMD (Ciprofloxacin)

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4	6					6
2	4	8	5	4	6	22
1		2	5	6	2	15
0.5						
0.25						
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	3.2	1.8	1.5	1.4	1.75	1.73
Mode	4	2	2	1	2	2
Min	2	1	1	1	1	1
Max	4	2	2	2	2	4
Range	2	2	2	2	2	3

Table 6. BMD (Erythromycin)

APPENDIX D

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
128						
64						
32						
16						
8						
4						
2						
1	9	5				14
0.5	1	5	3	7		16
0.25			7	2	7	16
0.125				1	1	2
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	0.95	0.75	0.325	0.413	0.234	0.547
Mode	1	0.5	0.25	0.5	0.25	0.5
Min	0.5	0.5	0.25	0.125	0.125	0.125
Max	1	1	0.5	0.5	0.25	1
Range	2	2	2	3	2	4

Table 7. BMD (Doxycycline)

MICs	lab 1	lab 2	lab 3	lab 4	lab 5	All labs
>256				4		4
256				6		6
128						
64		7				7
32		2				2
16		1	1		3	5
8			1		3	4
4	2		2		2	6
2	8		3			11
1			2			2
0.5			1			1
0.25						
0.125						
0.06						
0.03						
N	10	10	10	10	8	48
Geomean	2.4	52.8	4.05		10	
Mode	2	64	2	256	16	2
Min	2	16	0.5	>256	4	0.5
Max	4	64	16	>256	16	>256
Range	2	3	6	2	3	10

Table 8. BMD (Trimethoprim/Sulfamethoxazole)

BIBLIOGRAPHY

1. Food Safety and Inspection Service, U.S. Department of Agriculture. 1997. *FSIS/CDC/FDA Sentinel Study: the Establishment and Implementation of an Active Surveillance System for Bacterial Foodborne Diseases in the United States.* Report to Congress. Food Safety and Inspection Service, U.S. Department of Agriculture, Washington, D.C.

2. Tauxe, R.V. 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrial nations. In: Nachamkin I, Blaser MJ, Tompkins LS, editors.*Campylobacter jejuni*: current and future trends. Washington: American Society for Microbiology., 2:9-12.

3. King, E.O. 1962. The laboratory recognition of *Vibrio fetus* and closely related *Vibrio* species isolated from cases of human vibriosis. Ann. N.Y. Acad. Sci., 98:700-711.

4. Dekeyser, P.J., Gossuin-Detrain, M., Butzler, J.P., Sternon, J. 1972. Acute enteritis due to related *Vibrio*: first positive stool cultures. J. Infect. Dis., 125:390-392.

5. Smibert, R.M. 1984. Genus *Campylobacter*. In: Krieg, N.R., Holt, H.G., eds. Bergey's Manual of Systemic Bacteriology. v.1. Baltimore: Williams & Wilkins., 111-118.

6. Blaser, M.J., Taylor, D.N., Feldman, R.A. 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiol. Rev., 5:157.

7. Centers for Disease Control and Prevention. 1998. *1997 Final FoodNet Surveillance Report.* Foodborne Diseases Active Surveillance Network. Centers for Disease Control and Prevention, Atlanta, Ga.

8. Centers for Disease Control and Prevention. 1999. Incidence of foodborne illnesses: preliminary data from the Foodborne Diseases Active Surveillance Network (FoodNet)-United States, 1998. *Morbid. Mortal. Weekly Rep.* 48:189-194.

9. Council for Agriculture Science and Technology. Foodborne pathogens: risks and consequences. Ames(IA): The Council; 1994. Task Force Report No.122.

10. Williams, G., Faur, Y.C., Reimer, S.M. 1995. Quinolone Resistance in *Campylobacter jejuni.* ASM, 95th General Meeting, Washington, D.C.

11. Shoenfeld, Y., George, J., Peter, J.B. 1995. Guillian-Barre's Syndrome. New England J Med., 333:1415-7.

12. Mishu, B., Ilyas, A.A., Koski, C.L., Vriesendorp, F., Cook, S.D., Mithen, F.A., et al. 1993. Serologic evidence of previous *Campylobacter jejuni* infection in patients with the Guillian-Barre' syndrome. Ann Intern Med., 118:947-53.

13. Blaser, M.J., Wells, J.G., Feldman, R.A., Pollard, R.A., Allen, J.R., the Collaborative Diarrheal Disease Study Group. 1983. *Campylobacter* enteritis in the United States: a multicenter study. Ann Intern Med., 98:360-365.

14. Peterson, M.C., 1994. Clinical aspects of *Campylobacter jejuni* infections in adults. Wes J Med., 161:148-152.

15. Sorvillo, F.J., Lieb, L.E., Waterman, S.H. 1991. Incidence of campylobacteriosis among patients with AIDS in Los Angeles County. J Acquir Immune Defic Syndr Hum Retrovirol., 4:598-602.

16. Perlman, D.J., Ampel, N.M., Schifman, R.B., Cohn, D.L., Patton, C.M., Aguirre, M.L.,et al. 1988. Persistent *Campylobacter jejuni* infections in patients infected with the human immunodeficiency virus (HIV). Ann Intern Med., 108:540-546.

17. Blaser, M.J., Taylor, D.N., Feldman, R.A. 1984. Epidemiology of *Campylobacter* infections. In *Campylobacter Infection in Man and Animals* (J.P. Butzler, ed.), CRC Press, Boca Raton, FL,. 143-161.

18. Sjogren, E., Falsen, E., Kaijser, B., Lindbolm, G. 1996. *Campylobacter* species in faeces from healthy pets in Sweden isolated by filter technique. In *Campylobacter, Helicobacters*, and Related Organisms (D.G. Newell, J. Ketley, and R.A. Feldman, eds.), Plenum Press, New York. 471-473.

19. Blaser, M.J. 1985. *Campylobacter* species. In Principles and Practice of Infectious Diseases, 2nd ed. (G.L. Mandell, R.G. Douglas, Jr., and J.E. Bennett, eds.), John Wiley and Sons, New York, 1221-1222.

20. Jones, A., Harrop, C. 1981. A study of *Campylobacter* enteritis. J. Int. Med. Res., 9:40-43.

21. Cohen, D.I., Rouach, T.M., Rojol, M. 1984. A *Campylobacter* enteritis outbreak in a military base in Iseral. Isr. J. Med. Sci., 20:216-218.

22. Deming, M.S., Tauxe, R.V., Blake, P.A., et al. 1987. *Campylobacter* enteritis at a university: Transmission from eating chickens and from cats. Am. J. Epidemiol., 126:526-534.

23. Blaser, M.J. 1995. *Campylobacter* and related species. In: Mandell, G.L., Bennett, J.E., Dolen, R., eds. Principles and practices of Infectious Diseases, 4th ed, Churchill Livingstone, New York, NY, 1948-1956.

24. Stern, N.J., Line, J.E. 1992. Comparison of three methods for recovery of *Campylobacter spp.* from broiler carcasses. Journal of Food Protection., 55:663-666.

25. Rohrbach, B.W., Draughon, F.A., Davidson, P.M., Oliver, S.P. 1992. Prevalence of *Listeria monocytogenes, Campylobacter jejuni, Yersinia entercolitica*, and *Salmonella* in bulk tank milk: risk factors and risk of human exposure. Journal of Food Protection., 55:93-97.

26. Hudson, P.J., Vogt, R.L., Brondum, J., Patton, C.M. 1984. Isolation of *Campylobacter jejuni* from milk during an outbreak of campylobacteriosis. J. Infect. Dis., 150:789.

27. Lammerding, A.M., Garcia, M.M., Mann, E.D., Robinson, Y., Dorward, W.J., Truscott, R.B., et al. 1988. Prevalence of *Salmonella* and thermophilic *Campylobacter* in fresh pork, beef, veal, and poultry in Canada. Journal of Food Protection., 51:47-52.

28. Kapperud, G., Skjerve, E., Bean, N.H., Ostroff, S.M., Lassen, J. 1992. Risk factors for sporadic *Campylobacter* infections: results of a case-control study in southeastern Norway. J. Clin. Microbiol., 30:3117-21.

29. Humphrey, T.J., Henley, A., Lanning, D.G. 1993. The colonization of broiler chickens with *Campylobacter jejuni*; some epidemiologic investigations. Epidemiol. Infect., 110:601-607.

30. Kapperud, T.J., Skjerve, E., Vik, L., Hauge, K., Lysaker, A., Aalmen, I, et al. 1993. Epidemiological investigation of risk factors for *Campylobacter* colonization in Norwegian broiler flocks. Epidemiol. Infect., 111:45-55.

31. Kazwala, R.R., Collins, J.D., Hannan, J., Crinion, R.A.P., O'Mahony, H. 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commercial poultry production. Vet. Rec., 126:305-306.

32. Pearson, A.D., Greenwood, M., Healing, T.D., Rollins, D., Shahamat, M., Donaldson, J., et al. 1993. Colonization of broiler chickens by waterborne *Campylobacter jejuni*. Appl. Environ. Microbiol. 59:987-996.

33. Stern, N.J., Clavero, M.R.S., Bailey, J.S., Cox, N.A., Robach, M.C. 1995. *Campylobacter spp.* in broilers on the farm and after transport. Poult. Sci., 74:937-41.

34. Izat, A.L., Gardner, F.A., Denton, J.H., Golan, F.A. 1988. Incidence and levels of *Campylobacter jejuni* in broiler processing. Poult. Sci. 67:1568-1572.

35. Acuff, G.R., Vanderzant, C., Hanna, M.O., Ehlers, J.G., Golan, F.A., Gardner, F.A.. 1986. Prevalence of *Campylobacter jejuni* in turkey carcasses during further processing of turkey products. Journal of Food Protection., 49:712-717.

36. Blaser, M.J. 1990. *Campylobacter* species. In: Principles and practice of infectious diseases. Mandell, G.L., Douglas, R.G., Bennett, J.E., editors. 3rd ed. New York: Churchill Livingstone., 194:1649-1658.

37. Segreti, J. 1992. High level quinolone resistance in clinical isolates of *Campylobacter jejuni*. J. Infect. Dis. 165:667-670.

38. Endtz, II. P., Ruijus, G.J., van Klingeren, B., Jansen, W.H., van der Reyden, T., Mouton, R.P. 1991. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J. Antimicrobial Chemotherapy., 27:199-208.

39. Piddock, L.J.V. 1995. Quinolone resistance and *Campylobacter spp.* Antimicrob. Agents. Chemotherapy., 36:891-898.

40. Murphy, G.S. Jr., Echeverria, P., Jackson, L.R., Arness, M.K., LeBron, C., Pitarangsi, C.
Ciprofloxacin and azithromycin-resistant *Campylobacter* causing traveler's diarrhea in U.S. troops deployed to Thailand in 1994. Clin. Infect. Dis., 22:868-869.

41. Food Safety and Inspection Service, U.S. Department of Agriculture. *1997. Food Safety Research Agenda, Directions for the Future.* Food Safety and Inspection Service, U.S. Department of Agriculture, Washington, D.C.

42. Gaudreau, C., Gilbert, H. 1997. Comparison of disc diffusion and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni subsp. jejuni* and *coli.* J. Antimicrobial Chemotherapy., 39:707-712.

43. Huang, M.B., Baker, C.N., Banerjee, S., et al. 1992. Accuracy of the E-test for determining antimicrobial susceptibilities of *Staphylococci, Enterococci, Campylobacter jejuni* and Gram negative bacteria resistance to antimicrobial agents. J. Clin. Microbiol.
30:3242-3248.

44. Huysmans, M.B., Turnidge, J.D. 1997. Disc susceptibility testing of thermophilic *Campylobacters.* Pathology., 29:209-216.

45. Aarestrup, F.M., Nielsen-Moller, E., Madsen,M., et al. 1997. Antimicrobial susceptibility patterns of thermophilic *Campylobacter spp.* from humans, pigs, cattle and broilers in Denmark. Antimicrobial Agents and Chemotherapy., 41:2240-2250.

46. Svedhem, A., Kaijser, B., Sjorgen, E. 1981. The occurrence of *Campylobacter jejuni* in fresh food and survival under different conditions. J. Hyg., 87:421-425.

47. Vogt, R.L., Little, A.A., Patton, C.M., Barrett, T.J., Orciari, L.A. 1984. Serotyping and serology studies of campylobacteriosis associated with consumption of raw milk. J. Clin.Microbiol., 20:998-1000.

48. Mafu, A.A., Higgins, R., Nadeau, M., Couisineau, G. 1989. The incidence of *Salmonella, Campylobacter,* and *Yersinia enterocoliticia* in swine carcasses and the slaughterhouse environment. J. Food Prot., 52:642-645.

49. Casanovas, L., de Simon, M., Ferrer, M.D., Arques, J., Monzon, G. 1995. Intestinal
carriage of campylobacters, salmonellas, yersinias, and listerias in pigeons in the city of Barcelona. J. Appl. Bacteriol., 78:11-13.

50. Jacobs-Reitsman, W.F., Bolder, N.M., Mulder, R.W.A.W. 1994. Cecal carriage of *Campylobacter* and *Salomella* in Dutch broiler flocks at slaughter: a one year study.Poultry Sci., 73(8):1260-1266.

51. Jacobs-Reitsman, W.F., van de Giessen, A.W., Bolder, N.M., Mulder, R.W.A.W. 1995. Epidemiology of *Campylobacter spp.* at two Dutch broiler farms. Epidemiol. Infect., 114:413-421.

52. Pearson, A.D., Greenwood, M.H., Feltham, R.K., Healing, T.D., Donaldson, J., Jones, D.M., et al. 1996. Microbial ecology of *Campylobacter jejuni* in a United Kingdom chicken supply chain: intermittent common source, vertical transmission and amplification by flock propagation. Appl. Environ. Microbiol., 62:4614-4620.