ECOLOGY OF CAMPYLOBACTER COLONIZATION IN POULTRY:
ROLE OF MATERNAL ANTIBODIES IN PROTECTION
AND SOURCES OF FLOCK INFECTION

DISSERTATION

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School of The Ohio State University

By

Orhan Sahin, D.V.M., M.S.

The Ohio State University
2003

Dissertation Committee:

Professor Qijing Zhang, Advisor

Professor Teresa Y. Morishita

Professor Linda J. Saif

Professor Yehia M. Saif

Approved by

Department of Veterinary Preventive Medicine
ABSTRACT

*Campylobacter jejuni*, a gram-negative organism, is a leading bacterial cause of human foodborne enterocolitis worldwide. Although poultry are considered the major reservoir for this human pathogen, the ecology of *Campylobacter* in chicken flocks is poorly understood, hampering the design of effective intervention strategies at the preharvest stage. In the first part of this project, the prevalence, antigenic specificity, and bactericidal activity of poultry *Campylobacter* maternal antibodies were investigated. High levels of specific antibodies were detected in egg yolks, sera of broiler breeders, and young broiler chicks, as maternally-derived antibodies. These antibodies were against multiple outer membrane components of *Campylobacter*, and active in antibody-dependent complement-mediated killing of *C. jejuni*. These findings indicated the widespread presence of functional *Campylobacter* antibodies in the poultry production system, and prompted us to conduct the second part of the project to determine the effect of *Campylobacter* maternal antibodies on the colonization of young chickens by *C. jejuni* using challenge studies. Laboratory inoculation of commercial broilers of different ages showed that the onset of colonization occurred much sooner in birds challenged at the age of 21-days (which are naturally negative for *Campylobacter* antibody) than it did in the birds inoculated at 3-days of age (which are naturally positive for *Campylobacter* maternal antibody). Also, challenge experiments using laboratory-raised specific
pathogen-free (SPF) chickens demonstrated that significant decreases in the percentage of colonized chickens were observed during the first week post inoculation in 3-day old SPF chicks with *Campylobacter* maternal antibody as compared with 3-day old chicks without *Campylobacter*-specific maternal antibody. These results indicated that *Campylobacter* maternal antibodies play a partial role in protecting young chickens against colonization by *C. jejuni*.

In the last part of this study, we investigated the ability of *Campylobacter* to penetrate eggshells, its survival within eggs, and its prevalence in eggs from various sources to determine if vertical (egg-borne) transmission of *Campylobacter* occurred. Using a temperature differential method, it was shown that *Campylobacter* was either unable to penetrate the eggshell or did not survive up to 48 h inside the incubating eggs. *C. jejuni* survived for up to 14 days in eggs stored at 18°C following injection into the egg yolk; however, viability of the bacterium was dramatically reduced when injected into the albumen or the air sac. Although *Campylobacter* was detected in freshly-laid eggs obtained from an SPF flock colonized by *C. jejuni*, storage of eggs for 7 days at 18°C resulted in lack of detection of the organism. No *Campylobacter* was detected from 500 eggs laid by *Campylobacter*-colonized commercial breeders, and 1,000 eggs obtained from a commercial hatchery, suggesting that vertical transmission of *Campylobacter* through the egg is rare, and is unlikely to result in chicks infected with *Campylobacter*. 
Dedicated to my beloved family
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VITA

May 1, 1972 ……………………  Born-Gaziantep, Turkey

1988-1993………………………  D.V.M. Ankara University,
Ankara, Turkey

1996-1997………………………  M.S. The Ohio State University,
Columbus, Ohio, USA

1998-present……………………  Graduate Research Associate,
Food Animal Health Research Program,
Department of Veterinary Preventive Medicine,
The Ohio State University, OH, USA

ACADEMIC HONORS AND AWARDS


• Second best poster Award in OARDC Annual Conference, Ohio, U.S.A. 2000

• Best Poster Award in Comparative Gastroenterology, the 82th Annual Conference
  of Research Workers in Animal Diseases, St. Louis, U.S.A. 2001
• Membership of Delta Chapter of the Phi Zeta Honor Society, U.S.A. 2000 to present
• Membership of the American Society for Microbiology, U.S.A. 2000 to 2003
• Membership of Conference of Research Workers in Animal Diseases (CRWAD), U.S.A. 2001 to present

PUBLICATIONS

Research Publications


Published Abstracts


FIELDS OF STUDY

Major Field: Veterinary Preventive Medicine

Studies in Food Safety, Clinical Microbiology, Molecular Epidemiology
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CHAPTER 1

LITERATURE REVIEW

1.1. The Genus Campylobacter: Taxonomy and general description. At present, the genus Campylobacter contains 14 species, with Campylobacter fetus being the type species (159, 223). Based on recent extensive DNA-rRNA hybridization studies and 16S rRNA sequencing data, the family Campylobacteraceae was found to represent a diverse but phylogenetically distinct group, rRNA superfamily VI, within the group of gram-negative bacteria. This lineage is also known as the epsilon division of the Proteobacteria, and comprises rRNA homology groups I (Campylobacter and Bacteroides ureolyticus), II (Arcobacter), and III (Helicobacter and Wolinella succinogenes). Members of this lineage are characterized by their low chromosomal G+C content, inability to ferment carbohydrates, and microaerobic growth requirements.

The members of the genus Campylobacter (C. jejuni, C. coli, C. lari, C. upsaliensis, C. helveticus, C. fetus, C. hyointestinalis, C. mucosalis, C. concisus, C. curvus, C. showae, C. rectus, C. sputorum, and C. gracilis) are associated with a wide variety of diseases in humans and animals although some are considered commensals (157). Within the genus, three species (C. jejuni, C. coli, and C. lari) are known as thermophilic members of the genus and of clinical significance as they are the dominant
causative agents of human campylobacteriosis (97, 192). *C. jejuni* accounts for the majority of food-borne *Campylobacter* enteritis in humans, followed by *C. coli*, and to a lesser extent, by *C. lari* (97, 200).

As the name suggests, thermophilic *Campylobacter* spp. grow optimally at 42 °C on artificial media, although minimal growth occurs at 37 °C (191). They are relatively slowly-growing and fastidious, and require a microaerophilic atmosphere for optimal growth (175). *Campylobacter* cells are S-shaped spirally curved rods of 0.2 to 0.8 µm wide and 0.5 to 6.0 µm long, although cells may transform to spherical or coccoid forms as aged (191). The members of the genus are gram-negative, nonsporeforming, and possess a single polar flagellum, which endows the organisms with a characteristic corkscrew-like or darting motility (191). They are unable to ferment or oxidize carbohydrates, and thus energy is derived from the degradation of amino acids or tricarboxylic acid cycle intermediates (191).

1.2. Epidemiologic and clinical aspects of *Campylobacter* infections in humans: The majority of cases of human campylobacteriosis are characterized by a self-limited watery and/or bloody diarrhea, however, serious complications such as reactive arthritis (Reiter’s syndrome), Guillain-Barre syndrome, osteomyelitis, nephritis, myocarditis, cystitis, pancreatitis, septic abortion, and bacteremia may occur infrequently (5, 19, 200). Deaths due to *Campylobacter* infection are rare but occur primarily in immunocompromised patients, infants, and the elderly. *C. jejuni*, the predominant cause of *Campylobacter* enteritis, is now the leading cause of bacterial food-borne diseases reported in the United States and many other developed countries (2, 5, 67). An estimated
2.1 to 2.4 million cases of human campylobacteriosis occur in the US annually, exceeding the combined total number of diarrheal illnesses caused by *Salmonella* and *Shigella*. *C. jejuni* also is frequently associated with diarrhea in patients <6 months of age in developing countries, where the incidence of *Campylobacter* infection is much higher (19, 43). The rate of *Campylobacter* isolation ranges between 5 and 20% in children with diarrhea from parts of Asia, Africa, and South America (155). In the developing world, *Campylobacter* can be isolated as often as rotaviruses and enterotoxigenic *Escherichia coli*, and usually more frequently than *Salmonella* and *Shigella* from children with diarrhea (59, 155, 216).

Most cases of human campylobacteriosis are sporadic in nature and outbreaks due to *Campylobacter* are rare (5). Sporadic cases of *Campylobacter* infections have different epidemiological characteristics than outbreaks. Although most outbreaks are associated with raw milk and contaminated surface water (65), the majority of sporadic illnesses result from mishandling or consumption of undercooked poultry or cross-contamination of foods with raw poultry meat during food preparation (45, 50, 80, 88, 153, 207). Sporadic infections are also occasionally associated with traveling abroad, consumption of beef and pork, drinking of untreated water and milk, and contact with dogs and cats (5, 87, 111, 153, 160). *Campylobacter* transmission from person-to-person is uncommon (153, 189). The majority of outbreaks occur during the spring and autumn, while sporadic *Campylobacter* infections are often encountered during the summer months (67). In developed countries although a person of any age can be infected with *Campylobacter*, infants (<1 year old) and young adults (15 to 30 years old) have the highest attack rates (19). Immature immunity and first time exposure to *Campylobacter* are likely to be the
cause of the high rate of *Campylobacter* enteritis in infants. On the other hand, improper food handling practices common among young adult men may be associated with the high rate of campylobacteriosis in this age group (5, 50). In contrast, in developing countries *Campylobacter* infections most commonly affect young children (<5 years old), and decline with age (19, 155). Inflammatory (bloody) diarrhea is much less common among patients in developing countries, and asymptomatic infection among adults is frequent (217).

The infectious dose of *C. jejuni* for humans is low, with 500 to 800 organisms being able to cause illness as determined by experimental human infections (17). As estimated from outbreak investigations, the usual incubation period after ingestion of *C. jejuni* is approximately 24 to 72 hrs, but this period could be as long as a week (19). The major clinical symptom is an acute diarrheal illness with severe abdominal cramping and fever. The diarrhea may initially be watery, but frequently becomes bloody (particularly in developed countries) as the disease progresses. The worst symptoms of the illness usually last for 24 to 48 hrs, and the disease gradually resolves over a week. Relapses, which are usually less severe than the first attack, may occur in 20% of affected patients who did not receive a treatment (19). Patients may excrete *Campylobacter* in their feces for several weeks after the clinical symptoms have disappeared unless treated with antibiotics (200).

1.3. Pathogenesis of *Campylobacter* infections in humans: Although *Campylobacter* has emerged as a leading cause of bacterial food-borne illness during the past decade, relatively little is known of the mechanism of disease production (114, 222,
As is the case with many other food-borne infections, the outcome of *Campylobacter* infection is likely to be influenced by both host (age, health status, preexisting immunity) and pathogen specific factors (5). As a food-borne bacterial pathogen, *C. jejuni* in association with food or water enters the body, survives the acidity of the stomach, and colonizes the distal ileum and colon (114). Following colonization of the mucus blanket and adhesion to the intestinal cell surface, *Campylobacter* perturbs the normal absorptive capacity of the intestine by damaging epithelial cell function via cell invasion and/or toxin production, or by inducing host inflammatory reactions (114, 243).

Although a number of pathogen-specific factors may contribute to the pathogenesis of *Campylobacter* infections, none of them has a formally proven role (114). Potential determinants of virulence include chemotaxis, motility, flagella, host cell invasion, toxin production, iron uptake, inflammation, lipopolysaccharide (LPS) production, and stress proteins (114, 243). Flagellum is the most intensively studied virulence determinant of *Campylobacter*, and it exhibits phase and antigenic variation (30, 78). Studies using defined mutants has shown that the flagellum is required for colonization (and to cause disease) in humans and animals (114, 147, 232). Another important virulence trait of *Campylobacter* is its ability to adhere to and invade epithelial cells (230). *Campylobacter* components involved in adherence and invasion include flagella, LPS, fimbrial filaments, several surface exposed proteins (e.g., PEB1, and CadF), and CiaB (a secreted protein with similarities to the members of bacterial type III secretion system (71, 117, 120, 167, 222). Although toxin production was proposed to be one of the mechanisms for *Campylobacter* virulence, apart from the cytolethal distending toxin (CDT), genes encoding toxins have not yet been isolated, and the toxin production
varies substantially among *C. jejuni* strains (172, 222, 230). *Campylobacter* strains produce LPS or lipooligosaccharide (LOS) as part of bacterial cell component, however, unlike most other bacterial LPS, *Campylobacter* LPS/LOS may contain sialic acid (230). It is thought that LOS structures on the surface of certain serotypes of *C. jejuni* (e.g., O:19, and O:41) may elicit an antibody response against gangliosides on host peripheral neural tissue via molecular mimicry, leading to an autoimmune disease known as GBS (144).

### 1.4. Immunity to *Campylobacter* infections in humans:

Epidemiological investigations and experimental volunteer studies involving humans and other mammals indicate that protective immunity may develop as a result of prior *Campylobacter* infection, which may be effective in protection against the subsequent disease manifestations but not necessarily against colonization (139, 228, 230). In developing countries, there is an age-related increase in both circulating and intestinal antibody levels, which appears to play an important role in the age-related decrease in symptomatic *Campylobacter* infections (20, 24, 132, 215). The widespread incidence of asymptomatic infection in the developing world suggests that multiple exposure to *Campylobacter* induces a broad-range protective immunity, however, the number of natural infections required to induce such cross-protective immunity is unknown (230). Similarly in industrialized nations, development of resistance to *Campylobacter* enteritis may frequently occur in individuals who are occupationally exposed to *Campylobacter* such as slaughterhouse workers, veterinarians, and farmers (23, 36, 104). In such groups, circulating antibody titers are also frequently raised (36, 104). Also, human and animal
experimental studies indicate that primary infection with *C. jejuni* provides homologous protection against illness, but not necessarily against colonization (12, 17, 27, 183). In human volunteers, the protection against experimental *Campylobacter* was especially well-correlated with the preexisting circulating and intestinal IgA levels (18).

The majority of patients with *Campylobacter* infection develop circulating antibodies directed against a number of *Campylobacter* antigens. Experimental studies using human volunteers and other mammals showed that serum antibody responses develop rapidly after the onset of disease (17, 21, 27, 183). The specific IgA and IgM titers usually peak at approximately 10 days after the onset of illness, whereas IgG levels may not peak until 3-4 weeks post-infection. Even though IgA and IgM responses decline rapidly, IgG antibody levels persist for several weeks. Similarly, a long-lasting (up to 1-2 years) serum IgG response is observed in humans following natural *Campylobacter* infection, although levels of circulating IgM and IgA decreases dramatically after the first two months of symptomatic infection (35, 36, 94, 213). Besides circulating antibodies, both natural and experimental infections with *Campylobacter* induce immunity at local sites. Due to the enteric nature of *Campylobacter* infections, specific antibodies in the gut are likely to play an important role in protective immunity. Specific mucosal anti-*Campylobacter* antibodies, especially IgA, have been detected in feces, urine, milk, and saliva (18, 35, 124, 241). However, little is know about the kinetics and specificity of the local mucosal antibody responses against *Campylobacter*. As determined by Western blotting, the major antigens recognized by human serum and/or mucosal antibodies include flagellin, the MOMP, LPS, a 38 kDa surface protein (probably the adhesion protein CadF), and an unidentified 29 kDa antigen (35, 102, 138, 146). However, the
protective nature of these antigens remains to be determined. In addition to humoral immune response, cellular immunity also appears to be important to limit and clear the infection since higher incidence, severity and relapse of Campylobacter infections occur in patients infected with human immunodeficiency virus (168, 230). However, apart from a study which showed that the association of C. jejuni with colonic epithelium induced the secretion of a proinflammatory cytokine IL-8 (83), characteristics of specific cellular immune responses against Campylobacter have not been investigated.

1.5. Management of Campylobacter infections in humans: Even though clinical symptoms such as the presence of a pre-disease period of fever without diarrhea, intense abdominal pain, or extreme weakness may favor a diagnosis of campylobacteriosis, the disease cannot be clinically distinguished from other acute enteric infections such as salmonellosis and shigellosis. A definitive diagnosis of Campylobacter enteritis is therefore based on detection of C. jejuni in the feces of patients with diarrhea (19, 22, 200). A rapid presumptive diagnosis can be made by visualizing the organism in stools by Gram’s stain or by darkfield or phase-contrast microscopy, but the sensitivity of the microscopy is variable. Although patients with Campylobacter enteritis elicit both systemic and intestinal immune responses to infection, the diagnostic value of serology is limited as a substantial rise in antibody usually occurs after symptoms have resolved and fecal excretion of the bacterium has ceased (19, 36, 94, 213). However, presence of specific antibodies indicates a previous exposure to Campylobacter, and can be used to measure of the extent of exposure to the organism. Also, serodiagnosis may be more valuable for retrospective diagnosis of Campylobacter enteritis in patients with late
complications such as GBS since in such patients the fecal cultures are usually
Campylobacter-negative (19).

The majority of Campylobacter infections are mild and therefore do not require
specific antimicrobial therapy other than oral replacement of fluid and electrolytes lost
through diarrhea and vomiting (200). However, antimicrobial treatment is warranted for
compromised persons or for patients with severe, increasing bloody diarrhea or fever.
Currently, erythromycin is the first antimicrobial agent of choice for treating
Campylobacter infection, but fluoroquinolones, and tetracyclines are also used as
alternative drugs. However, use of latter agents has been hampered severely as resistance
to these antibiotics develops rapidly following the treatment (200).

Since epidemiological observations and experimental studies in human volunteers
indicate that previous exposures to Campylobacter may lead to protective immunity, the
development of a vaccine to prevent Campylobacter in humans is feasible (190).
However, efforts to develop an effective vaccine have been hindered greatly by the lack
of knowledge on the pathogenesis and cross-protective antigens of Campylobacter (190).
Also, considering the development of possible late term immunological complications
such as GBS following Campylobacter infection, safety becomes a major consideration
(121). Therefore, a subunit vaccine may be preferable to a whole cell vaccine, however,
bacterial factors involved in the pathogenesis of these immunological sequelae have not
been well defined (121).

1.6. Campylobacter colonization in poultry: Commercial poultry are the major
natural reservoirs of C. jejuni, and up to 100% of broilers at slaughter-age may harbor the
organism (99, 101). The prevalence in commercial broiler flocks varies greatly depending on the age of birds (14, 15, 63, 113). *Campylobacter* is rarely detected in broiler chickens less than 2-3 weeks old under commercial production conditions (6, 14, 63, 101, 198, 210), although newly hatched chickens can be experimentally infected with *C. jejuni* (196, 208, 247). For the majority of commercial flocks, *Campylobacter* infection is usually detected after the third week of age. Once some birds become infected, *C. jejuni* spreads rapidly to most of the birds in the flock, which remain colonized up to slaughter, leading to carcass contamination at the processing plants (15, 63, 72, 101, 198). *Campylobacter* shedding in chickens varies by season, being highest in the summer (6, 63, 72, 100, 149, 207, 233, 234). Even though *C. jejuni* is highly prevalent in broiler chickens, some flocks remain free of *Campylobacter* throughout their life span (6, 15, 92, 210, 219, 234). *Campylobacter* is also highly prevalent in chickens raised on organic or free-range farms (82, 179), indicating that different production systems are equally vulnerable to invasion by this organism. Besides chickens, *Campylobacter* colonization also occurs in other domestic poultry species including ducks, turkeys, ostriches, and geese with little or no clinical consequences (7, 126, 226, 245).

Colonization of chickens by *C. jejuni* occurs primarily in the lower intestines where the organism is mainly found in cecal and cloacal crypts (1, 11, 135). However, the organism can also be recovered to a lesser extent from the small intestines and the gizzard, and infrequently from the liver, spleen, and gall bladder (1, 109, 141, 247). Unlike the infection in mammals (e.g. mice, swine, rabbit, monkey, and humans), in which *C. jejuni* can invade intestinal epithelial cells and cause pathologic changes (8, 31, 184, 185), *C. jejuni* infection in chickens has several distinct features. First, it appears
that *C. jejuni* does not adhere directly to epithelial cells, but mainly locates in the mucous layer of the crypts (11, 135). Second, no gross or microscopic lesions are induced in chickens. Third, invasion of the intestinal epithelium usually does not occur. These observations indicate that *C. jejuni* is well adapted to the poultry host, and may be seen as a normal enteric flora by the host. Once a broiler chicken becomes infected, large numbers of *C. jejuni* can be detected in its intestinal tract and excreted in feces for at least 12 weeks (up to $10^8$ CFU/g feces) without any apparent clinical consequences to the chicken host (109, 207, 209). However, cecal colonization may not always result in detectable shedding into the feces (1, 122). *Campylobacter jejuni* can also be isolated at a high rate from the crops of market-age broilers, and feed withdrawal prior to slaughter (a common commercial practice used to reduce fecal contamination of carcass) significantly increases the isolation frequency from crops (1, 28, 29, 239).

Artificial inoculation of chickens with *C. jejuni* has revealed a number of factors that affected cecal colonization by this organism. It has been known that *Campylobacter* colonization rate can be influenced by the dose of the inoculum (187,196, 197, 208, 247). The minimum dose of the organism required for colonization could be as low as 35 CFU/bird via oral gavage (Stern *et al.*, 1988), however, the minimal infectious dose varies depending on the age of the chickens and strains of *C. jejuni* used (109, 187). The infectious dose can also be influenced by the route of challenge. Although Young *et al.* (247) were unable to infect one-day old chicks with a single *C. jejuni* strain (ATCC 33291) via cloacal route, an earlier study by Shanker *et al.* (196) demonstrated that both 2-and 14-day-old chickens required approximately 100-fold higher inocula when challenged with oral gavage rather than by the cloacal route. Experimental studies also
showed that different *C. jejuni* strains have varying colonization ability in chickens (37, 187, 196, 197, 208). Replacement of one *C. jejuni* strain by another has also been observed in both natural and experimental colonization studies in chickens, which indicates the possible presence of dominant *Campylobacter* isolates with the ability to displace others (101, 122). Although multiple *C. jejuni* isolates with different serotypes and genotypes can frequently colonize chicken flocks during the same production cycle (101, 198, 212, 219), infection of a single chicken with more than one strain of *Campylobacter* is a rare observation (122). To determine the colonizing factors of *C. jejuni*, Meinersmann *et al.* (136) compared the antigenic profiles of congenic strains with different colonizing phenotypes. The study did not reveal consistent differences between the colonizing and non-colonizing *C. jejuni* strains. However, the investigators noticed the exclusive association of a 69 kDa protein with the colonizing strain. Studies using genetically defined mutants revealed that flagella, DnaJ (heat shock protein), CiaB (*Campylobacter* invasin antigen B), PldA (phospholipase A), and CadF (*Campylobacter* adhesin to fibronectin) of *C. jejuni* were involved in colonization of chickens (119, 147, 232, 250, 251).

There is conflicting data regarding the colonization susceptibility of chickens of different ages. Some studies have shown that older chickens (~ 2-5 weeks of age) were more susceptible to *C. jejuni* colonization than younger ones (a few days old) (109, 187), while others indicated that both were equally susceptible to *Campylobacter* colonization (196, 197). Colonization of chickens by *C. jejuni* can also be affected by host lineage of chicken breeds (116, 211). Stern *et al.* (211) compared the resistance of three crossbred commercial broiler chickens to colonization by *C. jejuni* and showed significant
differences in colonization rate of various crossbreed of birds by different *C. jejuni* strains.

Despite the commensal relationship, *Campylobacter* colonization in chickens induces both systemic and mucosal humoral responses (34, 143, 152, 178, 236). Following experimental infection of day-old chicks via oral gavage, production of specific circulating IgM and IgA antibodies reaches significant levels within 1-2 weeks of infection and usually peaks at weeks 4-6 post-infection, followed by gradual decreases as birds age (34, 143). In contrast, detectable levels of IgG responses develop later than IgM and IgA responses, peak at 8-9 weeks of infection, and persist for a longer period (34, 143). *Campylobacter* colonization also elicits a substantial immune response in naturally colonized chickens, and specific antibodies readily transfer from hens to their progenies as maternally-derived (188, 246). A wide variety of antigens are recognized by chicken serum, with flagellin being the most common (34, 178, 237). However, antigens inducing protective immunity still remain to be identified. To date, immunization attempts using killed whole-cell or subunit vaccines (e.g., flagellin based), or live vectors genetically engineered to express *Campylobacter* antigens have had little if any protective effect in chickens even when delivered with mucosal adjuvants (115, 149, 178, 236).

### 1.7. Sources and transmission of *Campylobacter* in poultry:

**Horizontal transmission:** Circumstantial evidence has been accumulated in favor of horizontal transmission from the environment as the most probable source of poultry infection by *C. jejuni*. Potential sources include old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, equipment and
transport vehicles, and farm workers. However, none of these suspected sources have been conclusively identified as the formal source of infection for broiler farms. This is because, in many cases, comparison of isolates from broilers and the environment by phenotypic or genotypic typing methods was not performed, leading to questions about the significance of these putative sources of infection (48, 72, 113, 181, 202, 214). In those studies where the isolates from various sources were typed, the poultry isolates were frequently found to be different from those obtained in the immediate vicinity of the chicken farms (101, 148, 169, 182, 212, 219, 220). In addition, C. jejuni was most probably detected in suspect sources after the broilers had become infected, suggesting that broilers, instead of being infected from environmental sources, might be the source of environmental contamination (14, 101, 113, 210). In many situations, it was very difficult to determine which events (the flock infection or environmental contamination) occurred first, because no study plan was included to monitor the direction of Campylobacter transmission.

Since C. jejuni is very sensitive to oxygen and drying, the organism is generally unable to grow in feed, litter or water under normal ambient conditions (92, 97, 113). The organism is usually absent in fresh litter or feed samples before broilers are infected (72, 92, 101, 165, 220). Used litter may become contaminated by C. jejuni and may play a role in maintaining C. jejuni in the farm environment (140). However, a recent study by Payne at al., in which Campylobacter isolates were typed using randomly amplified polymorphic DNA-PCR and 23S rRNA-PCR, (164) did not support the role of litter in the transmission of the organism to successive flocks in the same poultry house. In European countries, since broiler houses are usually cleaned and disinfected and the litter
is changed between consecutive flocks, litter seems an unlikely source of infection in commercial broiler productions (62). Also, a recent nationwide epidemiological study in the USA indicated that there were no marked differences in the prevalence and onset time of *Campylobacter* shedding among flocks on different grow-out farms having different practices of litter use (210). Due to its low moisture content, feed is an unlikely source for the introduction of *C. jejuni* into the broiler houses (62, 101, 220). Feed itself, however, can be contaminated from other sources such as feces in the chicken house (72).

Groundwater is frequently used for drinking water on poultry farms, and unchlorinated water has been implicated as the source of *C. jejuni* into broiler chickens (112, 165). Due to its microaerophilic characteristics and inability to grow below 31°C (81), *C. jejuni* is unlikely to propagate in environmental water. The presence of this organism in streams, rivers, groundwater, and drinking water is a sign of a recent contamination with feces of livestock or wild birds (106, 202). Therefore, it is more likely that water is a passive source of infection rather than a niche for growth of *C. jejuni*. Also, drinking water on poultry farms generally becomes positive with *C. jejuni* only after chickens are colonized, suggesting that drinking water is not an original source of contamination (15, 99, 101, 113, 220).

Insects (house flies, darkling beetles, cockroaches, mealworms) can act as mechanical vectors, and may transmit *C. jejuni* from animal reservoirs to chicken flocks (99, 101, 181, 194). Identical serotypes and genotypes of *Campylobacter* were isolated from both broilers and insects within broiler houses, however, the direction of spread was not determined (6, 14, 101, 182, 212). Insects in poultry houses were usually not positive for *C. jejuni* until the organism was isolated from broilers (14, 148). Therefore, the
possibility of insects as an original source of infection to broiler houses is small, but insects may carry the organism from one location to another within or between flocks (14, 72, 181, 194).

Several studies have shown that rodents (mice and rats) and other small wild animals such as raccoons can carry *C. jejuni* in their intestine, and thus these wild animals are considered likely sources of *Campylobacter* introduction into grow-out houses (6, 13, 112, 148). However, *C. jejuni* was not isolated from rodents found in the vicinity of broiler houses in other studies (72, 105). In a recent study, the persistence of some clones of *C. jejuni* during successive broiler flock rotations was suggested to be a result of survival of the organism in such reservoirs as rodents and insects that were able to evacuate the house during cleaning and disinfection and then return (171). However, many other investigators found no evidence of transmission of *C. jejuni* from the first flock to the following flocks via persistence of the organism in broiler houses (72, 101, 219, 220). Considering the limited access of rodents into broiler houses, and the effective vermin control programs in most commercial poultry production facilities, the role of rodents as a common source of infection for broiler flocks is questionable (63, 72).

*Campylobacter* has a wide distribution in wild birds (26, 38, 64, 103, 110, 162, 245) Owing to their great mobility, wild-living birds may spread *Campylobacter* to other animals and humans through fecal contamination of pastures, forage, and surface water. Wild birds in the vicinity of poultry production facilities are often found to be infected with *C. jejuni*, however, isolates from wild birds are usually different from those of chicken origin (6, 72, 148, 182). Since wild birds have a high carriage rate of *Campylobacter* in their intestines, they should be considered a potential risk for
transmission of organism into broiler flocks (48). The exact role of wild-living birds in the introduction of *Campylobacter* into broiler houses will require further studies involving comparison of isolates from broilers and wild birds by genotyping methods.

Presence of other farm animals on broiler farms including pigs, cattle, sheep, and fowl other than chickens has been found to be associated with an increased risk of *Campylobacter* infection in broilers (15, 72, 112, 182, 219, 220). Gregory *et al.* (1997) indicated that cattle were the single common factor among three broiler farms positive for *C. jejuni*. In that study, cattle were found to be concurrently infected with *C. jejuni*. In a follow up study, *C. jejuni* isolates from these cattle were shown to have the same *flaA* type as the isolates from the broilers on the same farm (212). Identical genotypes between cattle and broiler isolates from the same farm were observed in another study, and cattle were suggested to be a source of infection to the broilers on the farm (220). However, as it was pointed out by the authors (220), the mode of spread was not known, and could have been from broilers to the cattle. In other studies, *C. jejuni* isolated from cattle was found to differ from the isolates recovered from the broilers on the same farm (99, 101, 148, 182), disputing the role of cattle as a source of poultry infection. Nevertheless, it should be kept in mind that cattle, like sheep and other farm animals, have the potential to contaminate pastures and surface waters, which in turn may act as a source for broiler infection (107, 204). Similar to cows, pigs are also common carriers of *Campylobacter* (6, 72, 148). Tending pigs before entering broiler houses was indicated as a risk factor for *Campylobacter* colonization of chickens (112). Although earlier studies found pigs and broilers to be infected with the same serotype of *C. jejuni* (6, 182), recent studies using more discriminatory typing tools showed that pigs and broilers on the same farm were
usually infected with different strains of *C. jejuni* (101, 212, 219, 220). In another study, no significant association was found between colonization of broilers by *C. jejuni* and presence of pigs on the same farm (100). Also, pigs are generally infected with *C. coli* instead of *C. jejuni* (207, 220). Other farm animals such as sheep and horses, and cats and dogs can also be infected with *C. jejuni* (207), however, their potential role as a source of broiler infection has not been established.

Farm workers loading birds for transport to slaughter may carry *C. jejuni* from one flock to another if they move between different flocks without changing clothes and boots (15). The organism has been isolated from footbath water, farmer’s boots, and transport crates (6, 99, 210, 220). Therefore, it is reasonable to assume that *C. jejuni* may spread between broiler flocks and farms by the movement of personnel. However, a recent study showed that two adjacent broiler houses that lacked biosecurity procedures were colonized with different genotypes (determined by *fla* typing and 23S rRNA-PCR typing) of *C. jejuni* even though these houses shared equipment and the farmer worked in both houses using the same boots (148).

Overall, these observations indicate that *C. jejuni* is widespread in the intestinal tract of many wild and domestic animals and birds, and ubiquitous in the poultry production environment, which makes transmission from environment to the broiler houses likely. However, there are still unresolved gaps in understanding the transmission of *Campylobacter* into broilers from environmental sources. No single factor was found to be the major risk for infection of broilers (92). It is most likely that introduction of *C. jejuni* to broiler flocks is mediated by multiple sources.
Vertical transmission: As aforementioned, many investigators have suggested that horizontal transmission from environmental sources is the major source of *Campylobacter* infection for broiler flocks, and vertical transmission is unlikely. The reason underlying this prevailing theory is related to several observations. First, young broiler chickens usually lack *C. jejuni* before 2 or 3 weeks of age even though the chicks are hatched from eggs from infected parent flocks (6, 14, 195, 219, 220). Second, although broilers from the same parent flocks are colonized by *C. jejuni* in some production cycles, they may be free of *Campylobacter* in other cycles (98, 101). Third, broiler flocks are frequently infected with strains different from those of breeder flocks (39, 170, 220). Fourth, chicken flocks originating from the same parent flocks do not always show similar serotypes (15), but broilers from different hatcheries may be infected with the same clones (171). Finally, isolation of *C. jejuni* from eggs from naturally or experimentally infected chickens has been very difficult and rare (54, 195), and so far live *Campylobacter* cells have not been detected from hatcheries or young hatchlings (6, 40, 84, 101, 113, 195).

Despite the observations disputing the role of vertical transmission, increasing evidence suggests that vertical transmission of *C. jejuni* may occur from breeder flocks to broiler farms through the egg. Earlier studies showed that, even if at a low level, *C. jejuni* could be isolated from both the outer (54) and inner (195) surface of egg shells laid by naturally infected commercial layers or broiler breeders. We also detected *C. jejuni* in a small number of freshly laid eggs obtained from layer chickens which were experimentally infected with *C. jejuni*, when a pool of whole eggs were mixed in a blender and subjected to selective enrichment for isolation (186). Shane et al. (1986)
isolated the organism from both interior surface of egg shell and egg contents after swabbing feces containing \textit{C. jejuni} onto the surface of the eggs. Following experimental infections of eggs with \textit{C. jejuni} by either temperature differential method (42) or inoculation of egg albumen via direct injection (195), the organism was recovered from both the contents of unhatched eggs and from the newly hatched chicks. Thus, these observations (42, 195) plus a recent finding that “viable but not culturable” forms of \textit{C. jejuni} could be resuscitated by injection into the yolk sac of embryonated eggs (33) indicate that once \textit{C. jejuni} enters inside the eggs, it could survive there long enough to potentially infect the hatchlings. The ability of \textit{C. jejuni} to survive in egg yolk even in the presence of high levels of \textit{Campylobacter}-specific antibody for long time is probably related to lack of complement in the yolk.

Detection of \textit{Campylobacter} DNA in eggs and young hatchlings has been shown in several studies. Chuma \textit{et al.} (40) found that as high as 35 \% of newly hatched chicks had \textit{C. jejuni} DNA as determined by a DNA-DNA hybridization method. However, the investigators were unable to detect any live \textit{Campylobacter} organisms by the enrichment culture method, suggesting that there were no live \textit{Campylobacter} in the chickens, or the organisms were in a “viable but not culturable” state. Similarly, \textit{C. jejuni} DNA was detected in the cecal contents of newly hatched chickens and 18-day-old embryos by PCR and/or Southern blot hybridization but not by conventional culture with selective enrichment (41). Recently, Hiett \textit{et al.} (84) reported PCR detection of \textit{Campylobacter} DNA in fluff and eggshell samples from hatcheries, although the same samples yielded no live organisms when conventional culture methods were used.
Following experimental infection of Japanese laying quails with \textit{C. jejuni}, the organism was recovered from the egg shell surfaces and egg contents (133). Since no \textit{Campylobacter} was isolated from the shell surface of several eggs which had the organism in their contents, and since \textit{C. jejuni} was isolated from the liver, matured yellow follicles and lower oviduct of these laying quails, it was thought that the egg contamination with \textit{C. jejuni} was caused by a systemic infection of the quail’s reproductive tract. \textit{C. jejuni} has also been isolated from ovaries and oviduct of healthy laying chicken hens (32, 99). Camarda \textit{et al.} (32) compared several \textit{C. jejuni} isolates recovered from the intestinal and reproductive tracts of laying hens using genotyping methods (\textit{fla} typing and pulsed-field gel electrophoresis), and showed that identical \textit{Campylobacter} strains could colonize both sites. In addition, results suggested that the oviduct colonization with \textit{Campylobacter} was via an ascending infection from the cloaca, and that certain strains of \textit{Campylobacter} could colonize the oviduct better than others. However, the exact role of infected reproductive organs in the contamination of eggs requires further research. \textit{C. jejuni} was able to invade and survive in egg contents for at least 3 days following immersion of Japanese quail eggs into bacterial suspensions for 30 seconds (134). The same study also showed that the organism could survive up to 86 days at 4 °C following injection into egg yolk, suggesting that \textit{C. jejuni} could infect egg contents and survive there for a long time.

Strict adherence to biosecurity on poultry farms has had limited success in preventing the infection of broilers with \textit{C. jejuni} (14, 15, 198, 220). In addition, chickens housed under a protective laboratory environment still became colonized by \textit{Campylobacter} (128). Application of strict control measures in two different broiler
houses, such as cleaning and disinfection of the houses between successive cycles, did not prevent the broiler flocks from becoming colonized by *Campylobacter*, even though some reduction in the percentage of *Campylobacter* positive flocks was achieved (220). Similarly, strictly-followed high-standards of hygiene and biosecurity measures practiced before placement of day-old broilers and during the entire grow-out period by all personnel reduced the prevalence of *Campylobacter*, but it did not prevent some flocks from being colonized by *C. jejuni* (70). Thus, even when the likely sources of horizontal transmission are controlled, broiler chickens still become infected with *C. jejuni*, raising the possibility that vertical transmission of *C. jejuni* may occur.

Finally, another line of evidence for vertical transmission of *C. jejuni* from breeder flocks to the progeny comes from a few observations that the isolates from both the breeders and the broilers had the same sero- or geno-types. Pearson et al. (165, 166) performed multi-year studies on a highly populated broiler chicken farm and provided evidence for the involvement of both vertical and horizontal transmission (165). Once the conditions for horizontal transmission were under control, a pattern of intermittent shed positivity within the same broiler flock, and the lack of diversity of types isolated during the entire study period became apparent, which indicated a common source of *C. jejuni* introduced by vertical transmission (166). Furthermore, the isolation rate (42.9 %) of *C. jejuni* in market-age broilers supplied by hatchery B was significantly higher than that (17.6 %) in broilers supplied by hatchery A in the same study. In two instances, when both hatcheries were used to hatch chicks to stock the same farm flock, *C. jejuni* was found only in those sheds with chicks supplied by hatchery B. Together, the result suggested that there was a common source of infection to the broiler farm (166).
Recently, Cox et al. (47) compared C. jejuni isolates from breeders and their progeny, and showed that the isolates from both places were of the same clonal origin as determined by sequencing the short variable region of flaA. The investigators interpreted the result as cultural evidence for vertical transmission of C. jejuni. Despite the observations supporting the possibility of a low rate of vertical transmission, live Campylobacter organisms have not yet been detected in the contents of commercial eggs, young hatchlings, or hatcheries (10, 40, 54, 84, 186, 193, 195). Thus, the exact role of vertical transmission in introducing Campylobacter to broiler flocks remains to be answered in future studies.

In conclusion despite extensive studies, the sources of infection and routes of transmission of C. jejuni in the poultry reservoir are still poorly understood. In the cases that C. jejuni isolates are typed using molecular methods, it is apparent that great genetic diversity exists among Campylobacter strains from within a flock and among adjacent flocks on the same farm. Existence of both C. jejuni-free and Campylobacter-colonized flocks on the same farm further complicates the understanding of the ecological features of this important human pathogen. These findings illustrate the complexity of the dynamics of C. jejuni transmission on poultry farms. Based on the current knowledge, it appears that multiple routes, including both vertical and horizontal transmission, are involved in the original introduction of C. jejuni into broiler flocks. It is likely that there is not a single dominating source for Campylobacter transmission on broiler farms. Rather, diverse sources of infection may exist on different farms. Once a flock is infected, the extent of Campylobacter colonization in the broiler flock is likely to be influenced by host-related factors (e.g. immune status of the birds) and environmental
conditions in the production system (e.g. management practices, biosecurity measures, and the presence of other farm animals). Therefore, multiple means targeting different segments of the broiler production system may be required to effectively reduce or eliminate \textit{C. jejuni} infection in broiler flocks.

1.8. \textit{Campylobacter} in the food: Many foods of animal origin are frequently contaminated with \textit{Campylobacter} spp., primarily with \textit{C. jejuni} (97). Epidemiological studies found a significant association between \textit{Campylobacter} infection in humans and consumption of poultry meat, red meat, and raw milk (207). Particularly, handling and eating raw or undercooked chicken is currently considered the major cause of human infections with \textit{Campylobacter} (45, 97). The high prevalence of \textit{C. jejuni} in market-age commercial poultry is reflected in a corresponding high level of contamination of poultry carcasses at the processing plants, and poultry products on retail sale (68, 97, 191, 207). Despite the differences observed in the level and extent of colonization of broiler flocks by \textit{Campylobacter}, processing plants serve to disseminate \textit{Campylobacter} (via routine applications utilized in a slaughterhouse such as scalding, defeathering, evisceration, and chilling) to all processed carcasses (10, 96, 235). Thus, large numbers (up to 100\% during the summer months) of retail broilers can carry \textit{C. jejuni}, and skin and giblets have particularly high levels of contamination (5, 238). The numbers of \textit{Campylobacter} organisms in fresh poultry products range between $\log_{10} 2$ and $\log_{10} 5$ CFU/carcass (16, 96, 97). Although handling and processing such as freezing diminishes the level of contamination of poultry meat with \textit{Campylobacter}, the organism can persist in poultry products under commercial conditions of handling and storage until the consumption by
humans (191, 244). *C. jejuni* was able to survive for at least three months when whole carcasses were stored at -20 °C (76, 191). Besides chicken carcasses, meat products derived from turkeys, ducks, and geese are also often contaminated with *Campylobacter*, however, the extent and level of contamination are usually lower compared to the products from commercial broilers (79, 97, 248).

Unlike sporadic infections, outbreaks of *Campylobacter* enteritis have been predominantly associated with consumption of unpasteurized milk (90, 108, 173, 174, 207). *Campylobacter* can be isolated from feces of as high as 70% healthy dairy cows and up to 12% bulk tank milk samples (56, 91, 97). The mean level of contamination of *Campylobacter* in milk as determined by a most probable number (MPN) is calculated to be between 1 and 100 CFU/100 ml (91). Raw milk is most likely to be contaminated through fecal material, however, direct contamination of milk through mastitis by *Campylobacter* can also occur (93, 97, 161). Milk products such as cheese and yogurt are not known to be contaminated with *Campylobacter* (9, 49). *Campylobacter* enteritis due to milk can effectively be avoided by proper pasteurization and prevention of recontamination after the heat treatment (97).

Although up to 90% of meat-producing cattle may excrete *Campylobacter* in their feces, numbers of *Campylobacter* on beef are reported to be small, and beef is not strongly implicated in human *Campylobacter* enteritis (97, 97, 204). However, several studies found that many of the serotypes of *C. jejuni* isolated from cattle were similar to those found in human disease, implicating beef products as a source of *Campylobacter* infection for humans (52, 69). Also, several studies reported up to 5% prevalence of *Campylobacter* in beef samples from retail outlets (207). Similar to beef, the level of
Campylobacter carriage on lamb and pork are low, and they do not appear to be a major source of human Campylobacter infection (97).

Non-chlorinated or contaminated ground and surface water are implicated as sources of Campylobacter outbreaks in humans (58, 68, 106). In Scandinavian countries, where streams appear to be clean, between 1.2 and 170 cases of Campylobacter infection per 100,000 people are estimated to occur due to exposure to contaminated water (106). In United Kingdom, Campylobacter outbreaks due to drinking water are usually confined to private water supplies, which are likely to be contaminated with animal waste (106).

As a result of presence of Campylobacter in water, shellfish may become contaminated by the organism. Campylobacter infections in humans due to consumption of raw clams and Pacific oysters have been reported (97). Campylobacter has been isolated from a variety of seafood, with a relatively high isolation rate of C. lari from these products. In one study, Campylobacter was isolated from 69% of batches of mussels and from 27% of batches of oysters (61). In another study, 47% of cockles, mussels, and scallops shortly after harvesting were found to be contaminated with Campylobacter (240). In the United States, Campylobacter was isolated from 21% of fresh crab meat from 12 different crab processing plants (177). It appears that consumers of raw shellfish obtained from shellfish beds located near sewage effluents, farmland runoffs, and waterfowl reservoirs (particularly gulls) are at an increased risk of contracting Campylobacter infection (97).

Vegetables can occasionally be contaminated with Campylobacter via the application of natural fertilizers, wild birds and farm animals, and contaminated surface water (97). Campylobacter was detected in (up to 3%) lettuce, radish, green onion,
parsley, and potato from the farmer’s outdoor markets, although these vegetables from supermarkets were found to be free from *Campylobacter* in the same large scale survey study conducted in Canada (163). There is a single case of human campylobacteriosis outbreak found to be associated with eating lettuce or tuna salad (180). However, it was suggested that these foods were a vehicle of transmission from cross contamination rather than being the primary source of infection (180).

1.9. Detection of *Campylobacter*: Sensitive and reliable detection methods are essential for understanding the epidemiology and ecology of *Campylobacter* in animal reservoirs, and ultimately for the design of intervention strategies to control *Campylobacter* infections. Thermophilic *Campylobacter* spp. are fastidious and slow-growing, requiring a microaerophilic atmosphere (containing 5% O₂, 10% CO₂, 85% N₂) and elevated temperature (42 °C) for optimal growth under laboratory conditions (46, 55, 81, 199). Thus, culturing *Campylobacter* spp. from fecal material with a high level of background flora is cumbersome and time consuming (25, 130, 142, 201), and requires the use of special culture media and conditions. Usually a selective medium is necessary to recover *Campylobacter* from field samples. Some of the most commonly used ones are Skirrow, Preston, Karmali, modified charcoal cefoperazone deoxycholate agar (mCCDA), cefoperazone amphotericin teicoplanin (CAT) agar, Campy-CVA (cefoperazone vancomycin amphotericin), and Campy-Cefex medium. Depending on the type and origin of the samples, these media may give varying recovery rates of thermophilic *Campylobacter* spp. (25, 74). Both solid agar plates and liquid enrichment media contain a variety of different combinations of antibiotics to which thermophilic
Campylobacter spp. are intrinsically resistant, such as polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin (46). These antibiotics inhibit the growth of many background microbial flora present in fecal specimens, whereby facilitating the isolation of slowly-growing Campylobacter spp.

Since Campylobacter spp. are sensitive to oxygen levels above 5%, all selective media for these organisms contain various oxygen-quenching agents in order to neutralize the toxic effect of oxygen radicals on Campylobacter (46, 201). The commonly used oxygen quenching agents include several types of blood, charcoal, hematin, and a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate.

Depending on the type of specimen, selective media can be used either for direct plating or for an enrichment step followed by plating for Campylobacter colonies. Although an enrichment step in liquid medium followed by plating on solid agar plates is usually superior to direct plating alone for isolation of Campylobacter from processed foods in which bacteria are usually in relatively low numbers and/or in an ‘injured’ state (46, 97), this procedure may not always perform better than direct plating when testing fecal samples. Feces from chickens usually contain high enough numbers (up to $10^8$ CFU/g feces) of Campylobacter organisms to be detected by direct plating (142, 207).

When isolating Campylobacter spp. from animal feces, one should consider the species of animal tested and the sites of specimen collection. Even though fecal samples from cattle and sheep can be enriched prior to plating for better recovery rates (25, 203, 204), enrichment may greatly reduce the recovery rate of Campylobacter spp. from different sites of the intestinal tract of poultry and pigs (130, 142).
Usually typical *Campylobacter* colonies are visible on solid media after 48 hrs incubation (25, 46, 218), but it may take up to 72-96 hrs to observe visible colonies of some slow-growing strains (145). Depending on the media used, colonies of *Campylobacter* spp. may appear differently. If the agar is moist, typical colonies look grey, flat, irregular, and thinly spreading. Colonies may form atypical shapes (round, convex, glistening) especially when plates are dry (46, 145). Once *Campylobacter*-like colonies appear on plates, identification at the genus or species level should be performed. This requires the use of multiple tests. Presumptive identification of thermophilic *Campylobacter* spp. can be done according to colony morphology, typical cellular shapes (spiral or curved rods), and characteristic rapid darting motility as observed under the phase-contrast microscope. The most commonly used phenotypic tests for identification of *Campylobacter* to genus or species level include biochemical tests (catalase, oxidase, nitrate reduction, hippurate hydrolysis, indoxyl acetate hydrolysis), antibiotic susceptibility patterns (nalidixic acid, cephalothin), and growth characteristics at different temperatures (25 °C, 37 °C, and 42 °C) (157, 205).

Immunology-based detection methods such as enzyme immunoassays (EIA) can be used for direct detection of *Campylobacter* spp. in animal feces or processed food. These assays are commercially available in a very similar format to sandwich-ELISA assays, which use two different antibodies, to detect *Campylobacter* directly in field samples (60, 85) or after a selective enrichment step (86, 127). Examples of these commercial kits include VIDAS *Campylobacter* (bioMerieux), EIA-Foss *Campylobacter* (Foss Electric), and ProSpecT (Alexon-Trend). EIAs are not as sensitive as culture methods for detecting *Campylobacter* spp., and are not suitable for testing samples in
which \textit{Campylobacter} spp. are suspected to be in low numbers. They are more rapid than traditional culture methods and can be automated for easy handling. Thus the EIA assays may be good choices for large-scale surveillance programs that deal with a large numbers of samples and require high throughput screening methods.

PCR-based methods are commonly used for the detection and identification of food borne pathogens including \textit{Campylobacter}. PCR allows exponential amplification of the targeted sequences within a short period of time, which permits the rapid detection of low numbers of organisms. PCR primers directed to conserved regions are usually used for general detection, while primers designed from variable regions can be used for differentiation of species or strains. A variety of PCR assays targeting genus or species specific sequences have been developed to detect and identify \textit{Campylobacter} in food (4, 53, 73, 131, 227, 242), feces (41, 89, 95, 176, 225) and environmental samples (214, 224).

PCR can be combined with other procedures, such as Southern blotting (3, 41, 118, 125, 154), reverse blot hybridization (44, 154, 221), and ELISA (73, 75, 137, 176, 227) to enhance detection efficiency. Reverse blot hybridization and PCR-ELISA employ specific capture probes immobilized onto membranes or microtiter plates, which are then hybridized with the PCR products amplified with labeled primers. Subsequent detection of the label is achieved using colorimetric systems. Both systems have the capacity of working with large numbers of samples, and can successfully detect, identify, and differentiate \textit{Campylobacter} species, depending on the design of the capture probes used. Since multiple probes specific for different species of \textit{Campylobacter} can be immobilized
onto a single membrane strip or in a microtiter plate, detection and differentiation of mixed populations can be simultaneously identified with a single test (44, 73, 154).

The main advantages of PCR are its sensitivity, specificity, and rapidness. However, PCR usually works best with pure bacterial cultures, and its performance on direct testing of field samples may be reduced dramatically due to the presence of PCR-inhibitors in animal feces and other types of samples. Purification of DNA from fecal, food, and environmental samples using a reliable method is essential for achieving satisfactory results with PCR. A number of techniques have been reported for the extraction of DNA from fecal or food materials prior to PCR. The most commonly used ones include phenol-chloroform extraction followed by ethanol precipitation (95, 118), commercial DNA purification kits (44, 51), immunomagnetic separation (53, 123, 151, 227), silicon dioxide extraction (89), column chromatography (225), and differential centrifugation (4, 41, 131, 176, 214, 224, 229). Despite the use of various purification methods, PCR inhibitors may not be completely removed from the reaction, decreasing the performance of PCR for detecting samples with low numbers of *Campylobacter*. An enrichment step is generally required prior to PCR (51, 73, 154), which not only increases the number of *Campylobacter* in the samples, but also dilutes the concentration of PCR inhibitors. Fecal samples from *Campylobacter*-shedding animals and humans may contain high enough numbers of the organisms to be detected directly without the need for an enrichment step (41, 89, 95, 129, 225).

1.10. Molecular typing of *Campylobacter*: Although detection and identification of *Campylobacter* species is the first step for epidemiological studies, a complete
understanding of the sources and modes of transmission of these pathogens in different hosts depends on genotyping of the isolates using molecular techniques. Prevention of human infections due to *Campylobacter* can be best achieved by successful determination of the sources and modes of transmission of these pathogens at the preharvest level, thereby permitting the implementation of effective intervention strategies. Molecular typing methods have provided unprecedented information on the epidemiology of *Campylobacter* both in animal reservoirs, particularly in poultry, and in humans. The most commonly used techniques include restriction fragment length polymorphism analysis of *flaA* sequences (PCR-RFLP), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and ribotyping (231). Besides these most commonly used molecular techniques, other genotyping methods based on sequence analysis can also be invaluable in epidemiological studies. For instance, sequence comparison of *cmp* gene, encoding the major outer membrane protein, from *Campylobacter* isolates of different origins has revealed that strains with identical *cmp* sequences had indistinguishable or closely related PFGE profiles (249). One advantage of sequence based typing tools is that they are highly reproducible and discriminatory.

Although molecular typing methods are usually highly discriminatory, sensitive, and reproducible, they largely suffer from a lack of standardization, making the comparison of data collected by different laboratories very difficult. To overcome this problem, a European Union-financed web site on the standardization of molecular typing methods for *C. jejuni* and *C. coli* has been developed (CAMPYNET Website [www.svs.dk/campynet.]). As with detection and identification methods, each of these
genotyping techniques has its advantages and disadvantages, and they usually give better discriminatory power if combined with serotyping or other molecular typing methods. PFGE and AFLP are commonly used, and seem to be the most discriminatory genetic typing tools as they are based on whole genome analysis, however, they both are time consuming and costly, and need to be standardized. On the other hand, \textit{fla}A typing is relatively simple, rapid and widely available, but it has less discriminatory power than PFGE and AFLP. Automated ribotyping (Riboprinter) has good discriminatory power and reproducibility, and is relatively standardized even though it is costly. Another whole genome-based molecular technique is RAPD, which despite being quick and cheaper than PFGE and AFLP, it lacks reproducibility.

As a result of widespread use of genotyping methods over the past decade, substantial progress has been made regarding the sources and transmission of \textit{Campylobacter} in poultry reservoirs even though there are still major gaps in the knowledge of the epidemiology and ecology of the organism in its animal hosts (148, 164, 170, 171). For instance, by using RAPD, used litter was excluded as a possible means of transmission of \textit{C. jejuni} in chicken flocks (164). Other studies utilizing \textit{fla} typing, PFGE, and RAPD have traced the infection of broiler chickens to many different sources, indicating the complexity of epidemiology of \textit{Campylobacter} in chicken farms (148, 169, 170, 220). Through use of PFGE, AFLP, and \textit{fla} typing, several investigators suggested that some genetic lineages were closely associated with a higher colonization potential of \textit{C. jejuni} in chickens (77, 171), indicating a possible value of genotyping methods in virulence studies. Apart from epidemiological investigations, molecular typing methods have also proven to be useful in taxonomic studies (57, 158). Recently,
Duim et al. showed that isolates of *C. lari* grouped into two distinct clusters using dendograms constructed from numerical analysis of the AFLP profiles, suggesting that this organism may comprise two genetically remote taxa (57).

Molecular typing methods are also indispensable to epidemiological studies of *Campylobacter* infections in humans, and to tracing the sources of outbreaks. Several epidemiological investigations found a clonal relationship in *Campylobacter* isolates from humans, and poultry, cattle and other sources, indicating the source of human infections (66, 77, 150). In addition, analysis of isolates from human patients using genotyping methods showed that all isolates from a single sample were almost always the same, indicating that human infection with more than one strain at a time is uncommon (206). Finally, molecular typing tools have also been used for tracing of the outbreaks caused by *C. jejuni*. During the investigation of a *Campylobacter* outbreak, a cafeteria worker with diarrhea was shown to be the most likely source of an outbreak occurring during a school luncheon as the food handler and the school children had the identical PFGE profiles (156).

1.11. References


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CHAPTER 2

PREVALENCE, ANTIGENIC SPECIFICITY, AND BACTERICIDAL ACTIVITY
OF POULTRY CAMPYLOBACTER MATERNAL ANTIBODIES

2.1 ABSTRACT

Poultry are considered the major reservoir for Campylobacter jejuni, a leading bacterial cause of human food borne diarrhea. To understand the ecology of C. jejuni and develop strategies to control C. jejuni infection in the animal reservoir, we initiated studies to examine the potential role of Campylobacter maternal antibodies in protecting young broiler chickens from infection by C. jejuni. Using an ELISA, the prevalence of C. jejuni antibodies in breeder chickens, egg yolks, and broilers from multiple flocks of different farms were examined. High levels of antibodies to the organism were detected in serum samples of breeder chickens and in egg yolk contents. To determine the dynamics of anti-Campylobacter maternal antibody transferred from yolks to hatchlings, serum samples collected from five broiler flocks at weekly intervals from one-day to 28- or 42-days of age were also examined using the ELISA. Sera from the one-day and seven-day-old chicks showed high titers of antibodies to C. jejuni. Thereafter, antibody titers decreased substantially and were not detected during the third and fourth weeks of age. The disappearance of Campylobacter maternal antibodies during 3-4 weeks of age
coincides with the appearance of *C. jejuni* infections observed in many broiler chicken flocks. As shown by immunoblotting, the maternally derived antibodies recognized multiple membrane proteins of *C. jejuni* ranging from 19 to 107 kDa. Moreover, an in vitro serum bactericidal assay showed that *Campylobacter* maternal antibodies were active in antibody-dependent complement-mediated killing of *C. jejuni*. Together, these results highlight the widespread presence of functional *Campylobacter* antibodies in poultry and provide a strong rationale for further investigation of the potential role of *C. jejuni* maternal antibodies in protecting young chickens from infection by *C. jejuni*.

2.2 INTRODUCTION

*Campylobacter jejuni* is the most common food borne bacterial pathogen of humans in the United States and other developed countries, and infection caused by this organism is characterized by self-limiting watery and/or bloody diarrhea (1, 14, 43). Epidemiological studies have also revealed that *Campylobacter* infection is associated with the development of Guillain-Barre’ syndrome, an acute neurological disease characterized by ascending paralysis of peripheral nerves, which may lead to respiratory muscle compromise and death (30). The majority of human *Campylobacter* infections result from consumption of undercooked chicken or food contaminated by raw chicken (1, 14, 45). Although *C. jejuni* colonizes a variety of wild and domestic animals and birds, commercial poultry is considered the major reservoir of human *Campylobacter* infections (14). Hence, reduction of the pathogen level in the poultry production system is essential for minimizing the threat of *C. jejuni* to public health.
In order to reduce or eliminate campylobacters from poultry, it is imperative to understand the ecological aspects of the infection in the reservoir. For the past several decades, a large number of farm-based studies have been performed to determine the epidemiological features of *C. jejuni* infections (14, 32, 37, 45). The general consensus is that *C. jejuni* is highly prevalent in chicken flocks, especially in chickens of more than 3 weeks old. The organism is carried in poultry intestinal contents in high numbers, leading to fecal contamination of chicken carcasses in processing plants (32, 37, 45). Despite this high colonization rate, infected chickens show little or no clinical signs of illness (37, 45). Sources of infection and modes of transmission for *C. jejuni* infection on poultry farms have not been well understood. Many studies suggest that horizontal transmission from environmental sources is the major mode of chicken flock infection by *C. jejuni* (11, 19, 32, 34, 45). However, several findings suggest that vertical transmission might also play a role in introducing *C. jejuni* from breeders into broiler flocks (9, 10, 13, 35, 39, 40). The complexity of *Campylobacter* transmission and the extensive nature of the colonization undermine the effectiveness of management-based intervention measures and highlight the need for alternative strategies, such as vaccination, to control *C. jejuni* infection in the poultry reservoir and consequently reduce the risk of human campylobacteriosis.

A general observation and, a unique characteristic of *C. jejuni* colonization in poultry, is that this organism is absent in chicks less than two weeks of age (32, 45), suggesting that young chicks may have intrinsic resistance to campylobacter colonization. However, the resistance mechanisms have not been defined. One possible contributing factor for this resistance may be the presence of *C. jejuni*-specific maternal
antibodies in young chicks. It is well-known that antibodies can be transferred from hens to their progenies. Maternal antibodies are usually sequestered from the maternal circulation by the developing oocyte, and subsequently transported from the egg yolk across the yolk sac membrane into the embryonic circulation (6, 20, 24). Transferred antibodies are predominantly of IgG class, while transfer of IgA and IgM is usually at substantially lower levels (20, 21, 24, 36). The level of maternal antibodies in young chicks peaks at 3-4 days after hatching, and thereafter gradually decreases to undetectable levels at two to three weeks of age (20, 21, 42). In the first week of life, when the level of circulating maternal antibody (Ab) and intestinal permeability of chicks are high, transport of circulating maternal Ab (mainly IgG) to the intestine occurs, which confers mucosal protection against infectious agents that colonize the intestinal epithelium. (16, 17, 28, 42, 44).

Currently, there is a considerable gap in our knowledge concerning poultry immune response to *Campylobacter* infection under natural conditions. There have been no reported studies examining the level or role of *C. jejuni* antibodies in chicken populations (including breeders and their progenies) on poultry farms. It is unclear if the *Campylobacter* maternal Ab is widely present in young broiler chickens on commercial farms, and if the maternally-derived antibodies would protect young chickens from *C. jejuni* infection. Elucidation of these aspects of poultry immune response to *C. jejuni* is critical for understanding the ecology of *Campylobacter* colonization in the poultry reservoir, and may provide new insights into the design of effective intervention measures to control *C. jejuni* infection in poultry. As a first step to examine the potential role of *C. jejuni* maternal Ab in protecting young chickens from *Campylobacter*
infection, here we examined the prevalence and levels of *C. jejuni*-specific antibodies in breeder flocks, egg yolks, and young broiler chickens, determined the antigenic specificity of these antibodies by western blotting, and evaluated the role of maternal antibodies in complement-mediated killing of *C. jejuni*.

### 2.3 MATERIAL AND METHODS

**Bacterial Strains:** *Campylobacter jejuni* strain ATCC 33291 and *Campylobacter coli* ATCC 33559 were obtained from the American Type Culture Collection, Rockville, MD. Other strains, including C1019, S2B, 81-176, 21190 and Turk, were described previously (2, 50). These strains were chosen for this study because they represented the collection of diverse isolates from human and poultry that are available in our laboratory. Cultures were grown in Brucella broth (BB) (Becton Dickinson, Sparks, MD) in anaerobic jars under microaerophilic conditions produced by CampyPack Plus gas-generating envelopes (BBL Microbiology System, Cockeysville, MD) at 42°C.

**Preparation of Campylobacter outer membrane components:** Outer-membrane components of *C. jejuni* were prepared using the ionic detergent *N*-lauroyl sarcosine as described previously by Blaser *et al.* (3). Briefly, cells grown in BB were harvested, washed in PBS, and then suspended in 10 mM Tris (pH 7.5) containing PMSF, a protease inhibitor (Pefabloc SC, Germany). The cells were then sonicated on ice using a VibraCell sonicator (Sonics and Materials Inc., Danbury, CT). The preparation was then centrifuged two times at 5,000 X g for 20 min to remove non-lysed cells. The supernatant was then centrifuged for 2 hr at 100,000 X g at 4°C, followed by the suspension of the pellet in 1%
(w/v) N-lauroyl sarcosine in 10 mM Tris (pH 7.5) and incubation at 25°C for 30 min on a shaker. This suspension was centrifuged again at 100,000 X g for 2 hr at 4°C. The pellet was washed and resuspended in 10 mM Tris (pH 7.5), and stored at – 80°C in small aliquots.

**Preparation of Glycine acid extract:** A crude mixture of surface proteins was extracted with 0.2 M glycine-HCl buffer (pH 2.2), as previously described by McCoy et al. (27). Briefly, bacteria were harvested from BB, washed twice in distilled water, and then suspended in 0.2 M glycine-HCl for 15 min at room temperature with stirring. The mixture was centrifuged for 25 min at 7,500 X g, and the supernatant (acid extract) was neutralized, dialyzed at 4 ºC against distilled water for 2 days, concentrated in a Speed-Vac (Savant Instruments, Holbrook, NY) for 1 h, and kept frozen at –80°C.

**Collection of serum samples and egg yolks:** Serum samples were collected from two parent breeder chicken farms, each of which was surveyed with seven different flocks (10 to 20 samples/flock). The age of the sampled birds ranged from 16 weeks to 14 months. Eggs were obtained from three local commercial layer farms for egg production (n=96) (Farms A, B, and C), and from a commercial hatchery for broiler chickens (n=107). To detect *Campylobacter* antibodies in eggs, yolk contents were separately collected and diluted 1:5 in PBS for storage at –80°C. To monitor the dynamic change of anti-*Campylobacter* maternal antibodies, sera were collected from five different broiler chicken flocks on two different farms at weekly intervals starting from one-day of age to 42-days of age.
Enzyme-Linked Immunosorbent Assay (ELISA): ELISA was used to determine the level of *C. jejuni*-specific IgG antibodies in breeder flocks, egg yolks, and commercial broiler chickens. Microtiter plates (Nunc-Immune Plate, Nunc, Denmark) were first coated with 100 µl of whole outer membrane components (ca. 60 ng/well) of *C. jejuni* in coating buffer (Sodium carbonate; pH 9.6) overnight at 25°C. Then, plates were incubated with a blocking buffer (PBS containing 2 % milk, 2 % bovine serum albumin, and 0.1 % Tween-20) at 37°C for 1 hr. Serum samples and egg yolks were diluted in the blocking buffer to 1:100, and then 100 µl of each dilution was added to individual wells. Duplicate wells were used for each sample. After incubation at 25°C for 2 hr, the plates were washed 3 times with the wash buffer (PBS containing 0.1% Tween-20). Goat-anti-chicken IgG conjugated to peroxidase (Kirkegaard & Perry) was diluted to 1:1000 in the blocking buffer and added to the wells (100 µl/well). After 2 hr incubation at 25°C, the plates were washed three times with the wash buffer and then the ABTS-peroxidase substrate was added (Kirkegaard & Perry). The OD values of individual wells were measured using an ELISA reader (Titertek Multiskan MCC\340) at 405 nm. A cutoff absorbance value for a positive sample was determined by adding 3 standard deviations to the mean absorbance value of negative controls. Sera and yolks from *Campylobacter*-negative SPF chickens, and serum samples from *Campylobacter*-negative 3-4 weeks-old broilers were chosen as negative controls. Yolk and serum samples from *Campylobacter*-colonized breeder chickens, which gave an ELISA OD value of 1.5 or higher, were used as positive controls.
**SDS-PAGE and Immunoblotting:** Outer membrane proteins and glycine-HCl extracts of *C. jejuni* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (22) with a 4% stacking gel and 10% separating gel. Protein samples were boiled in sample buffer at 100°C for 3 min prior to electrophoresis. The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, CA). The membrane blots were blocked with the blocking solution used in the ELISA assay for 2 hr at 25°C. Subsequently, the blots were incubated with and appropriate amount (400 µl for each membrane strip or 10 ml for a whole blot) of serum or yolk samples diluted to 1:100 in the blocking buffer for 2 hr at 25°C. After 3 washings with PBS containing 0.1% Tween-20, the blots were then incubated with peroxidase-conjugated goat-anti-chicken-IgG at a dilution of 1:1,000 for 2 hr at 25°C. The blots were then washed and developed with the 4 CN-peroxidase substrate (Kirkegaard & Perry).

To examine the chicken antibody response to the major outer membrane protein (MOMP) of *C. jejuni*, recombinant MOMP (rMOMP) was produced in *E. coli* and purified as described previously (50). The purified rMOMP was separated by SDS-PAGE and immunoblotted with chicken antibodies as described above.

**Serum Bactericidal Assay:** We chose to use *C. jejuni* strains 33291 (a human isolate) and 21190 (a poultry isolate) to assess the bactericidal activity of poultry maternal antibodies because they were isolated from different host species and were shown to be genetically divergent in a previous study (50). These two strains were grown in BB supplemented with *Campylobacter*-specific growth supplements (Oxoid,
Hampshire, England). Two-day-old cultures were diluted in broth to give approximately 2 $\times 10^4$ organisms/ml. Sera collected from two Campylobacter-negative SPF chickens were used as the complement source, which were confirmed for the lack of C. jejuni-specific antibodies by immunoblotting, filter sterilized using a 0.45 µm filter, and kept frozen in small aliquots at –80°C until use. To evaluate the role of Campylobacter maternal antibodies in complement-mediated killing of C. jejuni, serum samples from 10 one-day-old chicks, which contained high levels of maternal antibodies to C. jejuni as determined by ELISA and immunoblotting, were pooled and used as the Ab source in the bactericidal assay. Pooled sera derived from ten 21-day-old broilers that were negative for C. jejuni-specific Ab were used as control antibodies. In addition, a commercially available goat-anti-Campylobacter spp. Ab (Kirkegaard & Perry) was used as an additional control. These sera were inactivated at 56°C for 30 min prior to use to ablate complement activity. The bactericidal assay was performed in sterile microfuge tubes. Each reaction contained 50 µl of bacterial suspension, 50 µl of 1:5 diluted (in PBS) complement, and 10 µl of undiluted antibodies. Control tubes included i) bacteria plus complement only; ii) bacteria plus Ab only; and iii) bacteria plus PBS only. After the reactions were incubated at 37°C for 1 hr, 100 µl of the suspension from each tube was plated out onto Mueller-Hinton Agar (Becton Dickinson, Sparks, MD) plates, which were incubated for 2 days at 42°C under microaerophilic conditions. The CFUs were counted for each reaction, and % reduction in the number of live organisms was calculated using the formula: % reduction = [CFU (bacteria + complement only) - CFU (bacteria +Ab + complement)] / CFU (bacteria + complement only). For each antibody, the assay was repeated three times and the mean % reduction was presented in Table 2.1.
2.4 RESULTS

**Prevalence of *C. jejuni* antibodies in breeder chickens:** To assess if *C. jejuni* Ab is present in breeder chickens, serum samples collected from different breeder flocks were examined using an ELISA, in which the microtiter plates were coated with outer membrane proteins extracted from *C. jejuni* strain 33291. As shown in Figure 2.1, Panel A, all seven breeder chicken flocks from Farm I had very high levels of serum IgG antibodies to *Campylobacter*. In fact, every serum from these flocks (20 samples/flock) was positive for *Campylobacter* antibodies as determined by the ELISA. All seven flocks from Farm II were also positive for *Campylobacter* antibodies (Fig. 2.1, Panel A). Four flocks from Farm II had relatively lower, yet positive antibody level compared to other flocks in this farm. These results indicated that the levels of *Campylobacter* antibodies varied among breeder farms as well as among different flocks within a farm. Despite the variation, 100% of the serum samples from Farm II were positive as determined by ELISA. The age of the sampled birds ranged from 16 weeks to 14 months. However, there was no correlation between the antibody level and the age of the birds tested in this study.

**Prevalence of *C. jejuni* IgG antibodies in egg yolks:** To determine if antibodies to *C. jejuni* are present in eggs, yolk contents of eggs obtained from three layer farms and a hatchery (for broiler chickens) were tested for *Campylobacter* Ab using ELISA. All yolks from eggs of Farms A, B, and C were positive for antibodies to *C. jejuni* (Figure 2.1, Panel B). One hundred and five out of 107 eggs from the commercial hatchery for
broiler chickens were also positive for *Campylobacter* antibodies. These results indicated that *Campylobacter* antibodies are highly prevalent in egg yolks.

**Prevalence of *C. jejuni* maternal antibodies in young broiler chicks:** To determine the dynamic change of maternal antibodies in broiler chicks, serum samples were collected at weekly intervals from five flocks of broiler chickens and examined using the ELISA (Figure 2.2). High levels of *C. jejuni*-specific antibodies were detected in up to 7-day-old chicks. The levels of the antibodies dropped substantially at 14-days of age and reached background level at the third and fourth weeks of sampling. The absence of *Campylobacter* antibodies in the third and fourth weeks coincides with the onset of *C. jejuni* infection observed in many broiler flocks (32, 45). As observed with flock 1 (Fig. 2), *C. jejuni*-specific antibodies increased substantially at the fifth-week of age, and continued to rise at the sixth weeks of age (Figure 2.2). This recurrence of *Campylobacter* IgG antibodies during the fifth and sixth weeks of age could be due to the natural infection by this organism. However, isolation of *C. jejuni* was not done as part of this study. With other flocks (2-5; Fig. 2.2), no serum samples were collected and analyzed beyond the 4th week of age, because the focus of this experiment was to monitor the dynamic change of maternal anti-*Campylobacter* antibodies, which were not detectible after the third week of age.

**Antigenic specificity of poultry *Campylobacter* antibodies:** To examine the antigenic profiles recognized by the poultry *Campylobacter* IgG antibodies, outer membrane extracts of *C. jejuni* were separated by SDS-PAGE and immunoblotted with
representative egg yolks and serum samples derived from breeder chickens. It was shown that Ab responses in both serum and yolk samples were against multiple membrane components of *C. jejuni*, ranging from 19 to 107 kDa. There were considerable variations in the banding patterns recognized by antibodies in individual chickens or eggs (Figure 2.3). However, most of the serum and yolk samples showed a strong immune response to lipopolysaccharides (LPS) of *C. jejuni*, which appeared as a single diffuse band migrating below the 19 kDa marker. The majority of samples also contained antibodies to another unidentified antigen migrating at ca. 37 kDa. ELISA-negative-chicken sera and several SPF chicken sera showed no reaction to *C. jejuni* proteins on immunoblotting. As a positive control, *Campylobacter*-specific antibody raised in goat (Kirkegaard & Perry) was included in this experiment. Similar banding patterns to those recognized by the serum and yolk samples were observed with the positive control antibody, indicating that chicken antibody responses to this organism as measured by ELISA were specific to *Campylobacter*.

To test if the Ab response is strain- or species- specific, representative samples (breeder sera and yolks) were tested with antigens prepared from different strains of *C. jejuni* (ATTC 332291, C1019, S2B, 81-176, 21190, and Turk), and *C. coli* ATTC 33559. Results showed that individual samples reacted with multiple components in various strains of *C. jejuni* (Fig. 2.4). Cross-reactive antigens included the MOMP, flagellin, LPS, and other unidentified proteins (Fig. 2.4). Notably, the antibody response to *C. coli*, which is closely related to *C. jejuni*, was very limited in the chickens examined in this study, indicating the species specificity of the antibody response. This finding is
consistent with previous observations that the majority of *Campylobacter* infections in poultry are due to *C. jejuni*, instead of *C. coli* (38).

To determine the antigenic specificity of the maternally-derived *Campylobacter* antibodies, outer membrane extracts of *C. jejuni* were immunoblotted with serum samples from day-old broiler chicks (Figure 2.5, Panel A). The protein patterns recognized by the tested serum samples were similar to the banding patterns defined by the goat anti-*Campylobacter* Ab (Figure 2.5, Panel A, Lane 1), indicating the specificity of the poultry maternal antibodies to *Campylobacter*. As in the case with breeder serum and yolk samples, antibodies in the young broiler chicks were against multiple components of *C. jejuni*. Although there were variations in the banding pattern among different serum samples tested, most of these sera reacted strongly with several antigens, including the flagellin, LPS, and unidentified antigens migrating between 37 and 52 kDa.

To examine the maternal Ab response to surface-associated antigens of *C. jejuni*, antigens extracted by glycine-HCl were immunoblotted with sera from day-old chicks. Unlike the reactions to the sarcosinate-extracted outer membrane proteins, immunoblotting of the glycine-acid extracts of *C. jejuni* with the serum samples showed a single immuno-dominant band of approximate size of 58 kDa (Figure 2.5, Panel B), which was previously identified as the flagellin antigen (26). Since MOMP is an abundant membrane protein of *C. jejuni* and a potential candidate for vaccine design, the ELISA-positive serum samples of day-old chickens were further examined using rMOMP. As shown in Figure 2.6, the reactivity of individual serum samples differed, indicating the variability of the maternal Ab responses to MOMP in individual chickens.
Maternal antibody dependent bactericidal activity: To examine if anti-
*Campylobacter* maternal antibodies were involved in complement-mediated bactericidal activity, *C. jejuni* organisms were incubated with heat-inactivated sera from one-day (with maternal Ab) or 21-day old chickens (without maternal Ab) plus an exogenous complement source (Table 2.1). None of the heat-inactivated sera alone produced killing, indicating the requirement for complement. Two different strains, 33291 (a human strain) and 21190 (a chicken isolate) were evaluated with the bactericidal assay. Strain 33291 was virtually resistant to killing by the chicken antibodies in the presence of complement. However, this strain showed 85 % reduction in CFU counts when treated with the commercial goat anti-*Campylobacter* Ab. On the other hand, a substantial reduction (61 %) in CFU counts was observed when strain 21190 was incubated with the sera from one-day-old chicks (with maternal anti-*Campylobacter* Ab). However, no significant reduction was observed when sera from 21-day-old chicks (no anti-*Campylobacter* Ab) were used in the assay (Table 2.1). Interestingly, the goat-anti-*Campylobacter* Ab did not result in any killing of strain 21190 in the presence of complement. These results indicated that the chicken maternal Ab resulted in complement-mediated killing of *C. jejuni* in a strain-specific manner.

2.5 DISCUSSION

Although several studies have evaluated immune responses to *C. jejuni* in experimentally infected chickens or in a small number of commercial chickens (8, 29, 48), this work, to our knowledge, is the first extensive study documenting the prevalence of *C. jejuni* antibodies under natural conditions in the entire poultry production system.
including parent breeder chickens, eggs, and young broiler chickens. Results from this study clearly indicated that *C. jejuni* antibodies were highly prevalent in the poultry production system, possibly due to the extensive nature of *C. jejuni* colonization in the poultry reservoir. It was also shown in this study that the maternal antibodies in young broiler chickens reacted with multiple outer membrane proteins of *C. jejuni*, and were active in antibody-dependent complement-mediated killing of the organism. These findings underscore the need for further investigation on the potential role of anti-*Campylobacter* maternal Ab in protecting young chickens from infection by *C. jejuni*.

Serum immunoglobulins are readily transferred from hen serum to the egg yolk while the egg is still in the ovary. As the chick embryo develops, it absorbs the yolk immunoglobulins, which then appear in its circulation. The transferred antibodies are predominantly IgG, and transfer of IgA and IgM occurs at substantially lower levels (20, 21). During the first week after hatching, the permeability of the intestine is high, and thus high level of serum IgG can be transferred from the circulation into the intestinal tract, where it confers mucosal protection against enteric agents in young chickens (16, 17, 28, 42, 44). Unlike mammals in which maternal IgA derived from milk is a key component of maternal immunity against intestinal mucosal infections, young chickens mainly use maternal IgG from the circulation to combat intestinal infections (16, 17, 28, 42, 44). For these reasons, we chose to measure the serum IgG in young broilers to reflect the levels of maternal *Campylobacter* antibodies.

*C. jejuni* is generally absent in broiler flocks younger than 2 to 3 weeks of age (32, 45). It seems that there is an intrinsic resistance to natural spread of *C. jejuni* in
young chicks. This speculation is supported by the observation from an experimental study resembling the natural spread of *C. jejuni*, in which this organism was not detected in 50 birds until 11 days of age, even though these birds were commingled with a *C. jejuni* infected seeder bird starting from day two of age (18). Although mechanisms responsible for the intrinsic resistance have not been defined, one possible reason for the lack of *C. jejuni* infection in young broiler flocks may be related to the presence of *Campylobacter* maternal antibodies in the chicks. It has been known that maternal antibodies in young chicks confer protection against many enteric agents of poultry during the early stage of their life (16, 17, 28, 42, 44). It was also shown in experimental studies that *Campylobacter* antibodies in serum or intestinal secretions were associated with the resistance to *C. jejuni* colonization in various species of animals including chickens (5, 12, 47). Historically, the role of *Campylobacter* maternal antibodies may have been underestimated because many studies found that young chicks were susceptible to experimental challenge by *C. jejuni* (8, 41, 46). However, the oral gavage challenge method used in the previous studies did not resemble the natural process of *C. jejuni* transmission, and might overwhelm the protective role of maternal antibodies in the young chickens. There may be a threshold of infection dose required for successful colonization of *C. jejuni* in chickens (47), which is likely influenced by both the immune status and the genetic backgrounds of chickens. It is possible that the high prevalence of *Campylobacter* maternal antibodies in young chickens results in “flock immunity” against *C. jejuni* infection. This speculation needs to be examined in future studies.

Immunoblotting revealed that the chicken antibodies reacted with multiple outer membrane components of *C. jejuni*. The predominant *Campylobacter* antigens
recognized by the chicken antibodies appeared to be flagellin, LPS, and two unidentified proteins (37 kDa and 40 kDa, respectively; Figure 2.5). The flagellum of *C. jejuni* is required for in vivo colonization and induces a strong Ab response in both humans and animals (15). A potential protective role of anti-flagellin antibodies against *C. jejuni* infection has been observed in earlier studies involving humans (15, 31, 33) and chickens (7, 48, 49). Recently, a flagellin-based recombinant vaccine was shown in a mouse model to be protective against challenge by *C. jejuni* (23). These findings suggest that the flagellin protein of *C. jejuni* is an important immunogen. Therefore, a prominent antibody response to the flagellin antigen, as maternally-derived, in young broilers might contribute to the protection against colonization by *C. jejuni*. MOMP is the most abundant outer membrane protein of *C. jejuni* and is present in every strain of *Campylobacter jejuni* (3, 25). A previous study has revealed that MOMP sequences are divergent among different strains, which may lead to antigenic variation of the protein (50). Although convalescent human (31, 33) and rabbit (26) sera showed a strong immunoreactivity to MOMP, our study using both authentic and rMOMP showed variable reactivity of the chicken sera to this protein (Fig.2.6). This variability might be the reflection of antigenic variation of MOMP, because the testing strain (33291) used for the immunoblotting might have a MOMP that was antigenically divergent from the MOMP in those strains that infected the chickens.

Many *C. jejuni* strains have been shown to be susceptible to killing by normal or infected human sera, and the killing is mediated by both complement and specific Ab (4). Results from this study (Table 2.1) indicated that the chicken maternal antibodies significantly reduced the CFU counts when incubated with strain 21190 in the presence
of a complement source, but did not have any bactericidal effect on strain 33291. In contrast, the commercial goat anti-Campylobacter antibodies, which were generated by immunization with multiple strains of Campylobacter produced bactericidal effect on strain 33291, but not on strain 21190. It is unclear what contributed to the difference in the killing with the two strains. It is possible that the chickens examined in this study were infected by a Campylobacter strain that was similar to 21190 in antigens inducing complement-fixing antibodies, but was distinct from strain 33291. Consequently, the chicken maternal antibodies were active in the complement-mediated killing of strain 21190. Likewise, the commercial goat antibodies may contain complement-fixing antibodies that recognize antigens of strain 33291, instead of strain 21190, resulting in the inability to kill strain 21190. Together, these results suggest that the antibody-dependent complement-mediated killing of C. jejuni is strain-specific, requiring the presence of specific target antigens on the surface of the organism. It should be pointed out that the in vitro bactericidal activity of Campylobacter maternal antibodies does not necessarily mean that the maternal antibodies confer immune protection against C. jejuni in vivo. To confirm the protective role of Campylobacter antibodies, comprehensive experiments using young chickens with or without maternal Campylobacter antibodies and different challenge strains/doses are required in future studies.

2.6 ACKNOWLEDGMENTS

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2.7 REFERENCES


<table>
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<td>11 (5.7)</td>
<td>2.5 (2.3)</td>
<td>85 (8)</td>
</tr>
<tr>
<td>21190</td>
<td>61 (10.4)</td>
<td>7 (3.9)</td>
<td>-3.9 (3.4)</td>
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</tbody>
</table>

$^a$mean values of three independent experiments
$^b$pooled sera from one-day-old chickens (with maternal anti-$C.\, jejuni$ Ab)
$^c$pooled sera from 21-day-old chickens (no anti-$C.\, jejuni$ Ab)
$^d$commercial goat anti-$Campylobacter$ spp. Ab (Kirkegaard & Perry)

**Table 2.** 1 Bactericidal activity of poultry anti-$Campylobacter$ maternal antibodies.
Figure 2.1 Prevalence of anti-*C. jejuni* antibodies in sera of breeder chickens (A) and egg yolks (B) as determined by ELISA. A: Seven flocks on each of the two farms (I and II) were surveyed. Each bar represents the arithmetic mean ± the standard error of the mean of 20 (Farm I) or 10 (Farm II) serum samples in a single flock. B: Eggs from three different farms and one hatchery were tested for *Campylobacter* antibodies. Each bar represents the arithmetic mean ± the standard error of the mean. The number of tested eggs for each farm is labeled on the top of each bar. Negative controls (mean OD value = 0.179 ± 0.005) for panel A included 5 serum samples from 3-4 weeks-old *C. jejuni*-negative chickens. Five yolk samples (mean OD value = 0.220 ± 0.05) from SPF chickens were used as negative controls for panel B.
Figure 2.1 Prevalence of *C. jejuni* antibodies in sera of breeder chickens (A) and egg yolks (B) as determined by ELISA.
Figure 2.2 Dynamic change of serum IgG antibody to *C. jejuni* in broiler chickens. Serum samples collected from 5 different broiler flocks starting from day one to 28 (Flocks 2-5) or 42 days of age (Flock 1) were analyzed using the ELISA. Each bar represents the arithmetic mean ± the standard error of the mean of 23 serum samples.
Figure 2.3 Immunoblot analysis of serum IgG response to *C. jejuni*-outer membrane extracts in serum of breeder flocks (A), and in egg yolks (B). As positive control (Lane 1) *Campylobacter*-specific antibody raised in goat (Kirkegaard & Perry Laboratories Inc.) was used. Lanes 2-8: Individual breeder serum samples. Lanes 9 and 10: ELISA-negative chicken sera. Lanes 11-22: Individual yolk samples.
Figure 2.4 Immunoblot analysis of outer membrane components of thermophilic campylobacters using antibodies from an ELISA-positive egg yolk (Fig. 1B). Lanes 1: Low range protein standards (Bio-Rad). Lanes 2-7: *C. jejuni* isolates Turk, C1019, S2B, 81-176, 21190, 33291, respectively. Lane 8: *C. coli* ATTC 33559. Molecular weight markers are indicated on the left.
Figure 2.5 Immunoblot analysis of chicken serum maternal IgG antibody response to outer membrane antigens (A) or glycine-HCl extracts (B) of *C. jejuni* strain 33291 (a human isolate). (A) Lane 1: Goat anti-*Campylobacter* spp. Ab (Kirkegaard & Perry). Lanes 2-16: Individual serum samples collected from one-day-old chickens. Lane 1: A serum sample from 21-day old chicken. Molecular weight markers are indicated on the left. Fla indicates the position of flagellin. (B) Lane 1: A serum sample from 21-day-old chicken. Lanes 2-16: Individual serum samples from one-day-old chickens. The position of flagellin subunits (~60 kDa) is indicated by an arrow.
Figure 2.5 Immunoblot analysis of chicken serum maternal IgG antibody response to outer membrane antigens (A) or glycine-HCl extracts (B) of C. jejuni strain 33291 (a human isolate).
Figure 2.6 Immunoblot analysis of serum maternal IgG antibody response to recombinant major outer membrane protein of *C. jejuni* (strain 33291). Lanes 1-20: Individual serum samples from one-day-old chickens. The position of the rMOMP (ca. 45 kDa) is indicated by an arrow.
CHAPTER 3

EFFECT OF CAMPYLOBACTER-SPECIFIC MATERNAL ANTIBODIES ON THE COLONIZATION OF YOUNG CHICKENS BY CAMPYLOBACTER JEJUNI

3.1 ABSTRACT

Using laboratory challenge experiments, we examined whether Campylobacter-specific maternal antibody (MAB) plays a protective role in young chickens, which are usually Campylobacter-free under natural production conditions. Kinetics of C. jejuni colonization were compared by infecting 3-day old broiler chicks, which were naturally positive for Campylobacter-specific MAB, and 21-day old broilers, which were negative for Campylobacter specific antibody. The onset of colonization occurred much sooner in birds challenged at the age of 21-day than it did in the birds inoculated at 3 days of age, suggesting a possible involvement of specific MAB in the delay of colonization. To further examine this possibility, specific pathogen-free (SPF) layer chickens were raised under laboratory conditions with or without Campylobacter infection, and their 3-day old progenies with (MAB\textsuperscript{+}) or without (MAB\textsuperscript{−}) Campylobacter-specific MAB were orally challenged with C. jejuni. Significant decreases in the percentage of colonized chickens were observed in the MAB\textsuperscript{+} group during the first week as compared with the MAB\textsuperscript{−}
group. These results indicate that Campylobacter-specific MAB plays a partial role in protecting young chickens against colonization by *C. jejuni*. Presence of MAB in young chickens did not seem to affect the development of systemic immune responses following infection with *C. jejuni*. However, active immune responses to *Campylobacter* occurred earlier and more strongly in birds infected at 21-days of age than those infected at 3-day of age. Clearance of *Campylobacter* infection was also observed in chickens infected at 21 days of age. Collectively, these findings indicate that *Campylobacter* MAB is a contributing factor for the lack of *Campylobacter* infection in young broiler chickens in natural environments, and suggest the feasibility of use of immunization-based approaches for control of *Campylobacter* infections in poultry.

### 3.2 INTRODUCTION

*Campylobacter jejuni*, a gram-negative bacterium, is the most commonly reported bacterial cause of human foodborne illness in the United States and other developed countries. Each year, an estimated 2.1 to 2.5 million cases of human campylobacteriosis, characterized by watery and/or bloody diarrhea, occur in the United States (1, 5, 12). *Campylobacter* infection is also the most common antecedent of Guillain-Barre syndrome (GBS), a demyelinating disorder that causes acute neuromuscular paralysis of peripheral nerves and respiratory muscle compromise that may lead to death (25, 39). The majority of human *Campylobacter* infections result from consumption of undercooked poultry or other food products cross-contaminated with raw poultry meat during food preparation (8, 10, 16). Thus, reduction or elimination of poultry contamination by *C. jejuni* would greatly decrease the risk of human campylobacteriosis.
At present, no effective control measures are available for prevention of *Campylobacter* colonization of commercial broiler chicken flocks. Limited success of utilizing improved hygiene measures in reducing carcass contamination at slaughterhouses highlights the need for farm-based intervention methods to control *Campylobacter* (23). Due to the complexity of *Campylobacter* transmission and the ubiquitous distribution of the organism in poultry production environments, management-based methods such as strict biosecurity measures have had little success in preventing introduction of *C. jejuni* into the poultry flocks (3, 35). Therefore, alternative intervention strategies, such as vaccination, are needed to control *C. jejuni* infection in the poultry reservoir. However, little effort has been directed to the study of immunity in poultry to *Campylobacter* colonization. The nature of the protective immune responses against *C. jejuni* colonization in chickens is unknown.

A general observation, and a distinct characteristic of *C. jejuni* colonization in poultry, is that this organism is not detected in chicks less than 2-3 weeks of age under conditions of commercial broiler production (11, 17, 35). Infection of broiler flocks by *C. jejuni* usually starts from the third week and peaks at market-age (6-7 weeks). This unique ecological feature suggests that young chickens may have an age-related resistance to *Campylobacter* colonization. However, the resistance mechanisms have not been defined. Elucidation of the factors contributing to the lack of *Campylobacter* colonization is of particular interest as this may provide valuable information for designing strategies to reduce or even eliminate *Campylobacter* colonization in broiler chickens. One possible contributing factor for the resistance may be related to *Campylobacter*-specific maternal antibody (MAB) in young chicks. Previously, we have
documented the widespread presence of *Campylobacter*-specific antibodies in broiler breeders, eggs, and broiler chicks as maternally-derived (31). In fact, 100% of day-old hatchlings obtained from five different commercial broiler flocks were positive for MAB against *Campylobacter*. In all birds from each flock, high levels of circulating *Campylobacter*-specific MAB were detected during the first week of age. Thereafter, the levels of MAB dropped substantially at 14 days of age and reached background levels by the third and fourth weeks of age. Western blotting demonstrated that MAB reacted strongly with multiple components of the outer membranes of *C. jejuni* including flagellin, LPS and other unidentified proteins. Moreover, *Campylobacter*-specific MAB was shown to be effective in complement-mediated killing of *C. jejuni* isolates in a strain-dependent manner (31). These findings suggested that MAB may protect young chickens from colonization by *C. jejuni* and prompted this study to assess the protectiveness of *Campylobacter* MAB using more defined challenge systems.

Historically, the role of *Campylobacter* MAB may have been neglected since many studies found that young chickens were susceptible to colonization by *C. jejuni* following experimental challenges (7, 13, 33, 37). However, the actual level of MAB in these experimentally infected chicks was not measured, and the relatively high challenge doses might have overwhelmed any protection conferred by MAB. Thus, more defined experimental challenge studies with appropriate controls are necessary to better understand if maternal immunity is protective against *Campylobacter* colonization in young chickens. Toward this end, we conducted laboratory challenge studies to determine the effect of *Campylobacter*-specific MAB on the colonization of young chickens by *C. jejuni*. Infection of chickens with different doses and strains of *C. jejuni*
revealed that *Campylobacter*-specific MAB markedly delayed the onset of colonization and reduced the rate of horizontal spread of *C. jejuni* in young chickens. This finding indicates a partially-protective role of *Campylobacter* MAB and suggests that MAB is a contributing factor to the lack of *Campylobacter* colonization in chickens less than 2-3 weeks of age.

3.3 MATERIAL AND METHODS

**Bacterial Strains and Culture**: *Campylobacter jejuni* strains 21190 and S3B (both of poultry origin) were chosen for use in challenge experiments because of their ability to colonize chicken ceca. Also, these two strains are genetically diverse as determined by PFGE and sequencing of the *cmp* gene (41). Bacterial cultures were grown for 48 hrs in Mueller-Hinton (MH) broth in anaerobic jars under microaerophilic conditions produced by CampyPack Plus (BBL Microbiology System, Cockeysville, MD) at 42 °C.

**Challenge experiments using broiler chickens**: Day-old commercial broiler chickens were obtained from a local commercial hatchery. Birds were housed in wire-floored cages in steam-cleaned and formaldehyde-fumigated rooms and provided with unlimited access to feed and water. The feed (C-2-88, OARDC, The Ohio State University) was custom-made, *Campylobacter*-free, and without any animal protein or antibiotic additives. Prior to challenge, all birds were confirmed to be free of *Campylobacter* as determined by culturing of cloacal swabs. The overall design of the challenge experiments using commercial broiler chickens is presented in Table 3.1. Since
every 3-day-old broiler bird was positive for anti-Campylobacter MAB, we were unable to find MAB-negative broiler chicks from commercial sources for control groups. Thus, it was not feasible to determine the role of MAB via challenge studies using 3-day old commercial broiler chickens. However, it was known from our previous work that 21-day old broiler chickens were free from Campylobacter-specific antibody (31). Therefore, some broiler chickens were kept under laboratory conditions for three weeks and then used for the challenge experiments with C. jejuni. In experiment I, 3-day old (Group 1; with Campylobacter MAB) and 21-day old (Group 2; with no antibody to Campylobacter) broiler chickens (originated from the same hatch) were inoculated with a relatively low dose (3X10^2 CFU/bird) of C. jejuni strain S3B via oral gavage. Cloacal swabs were taken one day before challenge and every other day after the challenge until the end of the experiment to isolate Campylobacter. Also, on days 0, 7, 14, 21 and 28 post-challenge, a blood sample was collected from each bird, and the level of serum IgG specific for Campylobacter was determined by ELISA as described below. Experiment II was performed in a similar way to Experiment I except C. jejuni strain 21190 was used to infect both 3-day old and 21-day old broiler chickens.

**Challenge experiments using SPF chickens:** To further assess the protective effect of MAB on Campylobacter colonization using a more defined system, fertile eggs from specific pathogen-free (SPF) hens were purchased from Hy-Vac (Iowa), and hatched in the laboratory animal facilities of our department to establish SPF flocks free of Campylobacter. At the age of 15-weeks, the birds were divided into two flocks (A and B), each consisting of 15 pullets and 4 cockerels. The absence of colonization of these
birds with *Campylobacter* was confirmed by culturing of cloacal swabs. Once the hens started laying eggs, birds in flock A were orally inoculated with *C. jejuni* strain S3B (ca. 1X10^7 CFU/bird) at the age of 22-weeks, while birds in flock B remained uninfected to serve as negative control. Starting from the second week after the inoculation, eggs were collected from both groups and tested for the levels of *Campylobacter*-specific antibody by ELISA as described previously (31). When significant levels (an ELISA OD >1.0) of *Campylobacter* antibody were observed in egg yolks from flock A (ca. 4 weeks after inoculation), hatchlings were obtained from eggs of both flock A and B. The young hatchlings were tested for the level of serum antibodies specific for *Campylobacter*. As expected, the chicks hatched from the eggs of flock A were positive for *Campylobacter*-specific MAB, while the majority of chicks from the eggs of flock B were negative for the specific MAB. The hatchlings were housed in wire-floored cages and used for challenge experiments.

The overall design of the challenge experiments using SPF hatchlings is illustrated in Table 3.2. In experiment I, 3-day-old SPF chicks were divided into four groups, each of which consisted of 11-12 birds. Birds in Groups 1 and 2 originated from *C. jejuni*-infected hens (flock A), and thus had high levels of *Campylobacter*-specific MAB (MAB^+). In contrast, birds in Groups 3 and 4 were obtained from *Campylobacter*-free SPF hens (flock B) and were negative for any specific antibody to *Campylobacter* (MAB^−). To determine the effect of MAB on colonization with a homologous strain, the chickens in experiment I were challenged with strain S3B, which was used to infect the SPF hens in flock A. The chicks in Groups 1 and 3 were infected with a relatively low dose (5X10^3 CFU/bird) of S3B, while those in Groups 2 and 4 received a higher dose.
(5X10^5 CFU/bird) of the same strain via oral gavage. Cloacal swabs were taken once every other day during the first 2 weeks after challenge, and once per week for an additional 2-week-period. The swabs were used to isolate C. jejuni from feces. In addition, serum samples were collected from the birds one day before challenge, and on days 6, 14, 21, and 28 post-challenge for detection of Campylobacter antibody. Experiment II was performed in the same way as experiment I except that C. jejuni strain 21190 was used to infect chicks to determine the effect of MAB on colonization with a heterologous strain (Table 3.2). The challenge doses were 2X10^4 CFU/bird for Groups 1 and 3, and 2X10^6 CFU/bird for Groups 2 and 4, respectively. The birds in experiment II were monitored for two weeks longer than those in Experiment I (Table 3.2).

**Isolation of Campylobacter from cloacal swabs:** To enumerate Campylobacter cells in feces, cloacal swabs were suspended and serially diluted in MH broth. One hundred µl of each fecal suspension was directly plated onto MH plates containing Campylobacter-specific growth supplements (Oxoid). Following incubation for 2-3 days under microaerobic atmosphere at 42 °C, Campylobacter-like colonies were counted. The isolates were confirmed as C. jejuni using a species-specific PCR test (38). Both the percentage of colonized chickens and numbers of organisms shed in feces (CFU/g feces) in colonized birds were calculated, and used to determine the degree of protection conferred by MAB. The limit of sensitivity of this detection method as determined by recovery of the organism from artificially inoculated fecal samples was approximately 200 CFU/g feces.
**Antibody detection by ELISA:** Campylobacter-specific IgG antibodies in serum and yolk samples were measured using an indirect ELISA described previously (31). In the experiments involving broiler chickens, ELISA plates were coated with outer membrane components of *C. jejuni* strain 33291, whereas the plates were coated with outer membrane components of strain S3B in the experiments involving SPF chickens. For detection of *Campylobacter*-specific IgM and IgA antibodies, serum samples were diluted 1:100 in a blocking buffer containing 2 % BSA, and 2 % skim milk in PBS-0.1 % Tween-20. Goat anti-chicken IgM and IgA conjugated to peroxidase (Bethyl Laboratories, Montgomery, TX) were diluted 1:500 in the same blocking buffer for use as secondary antibodies. After a 30-min reaction with ABTS-peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), absorbance values (OD) at 405 nm were recorded using a spectrophotometer (Emax™, Molecular Devices, California). For serum IgG, a cutoff absorbance value of 0.270 was used to indicate a positive sample. This number was calculated by adding 3 standard deviations to the mean absorbance value of negative controls, which were 115 serum samples from 3-week old broiler chickens as determined previously (31).

**Serum Bactericidal Assay:** To assess bactericidal activity of sera obtained from 2-day old broilers or SPF White Leghorn chicks, serum bactericidal assay was performed using *C. jejuni* strains S3B and 21190 as described previously (31).

**Genetic characterization of Campylobacter isolates:** To verify that the *Campylobacter* isolates recovered from the experimental chickens originated from the
inoculated strains, genotyping of *Campylobacter* isolates was performed by sequencing of the *cmp* gene (coding for the major outer membrane protein) as described previously (41). The *cmp* alleles in strain S3B and 21190 (GenBank accession no. AY083463, and AF285141, respectively) are distinct from the known *cmp* alleles in other strains. The forward primer F3 (5’-ATGAAACTAGTTAAAACCTTAGTTTA-3’) and reverse primer R3 (5’-GAATTTGTAAAGAGCTTGAAG-3’) were used in PCR to amplify the *cmp* gene from various isolates. The amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen) and subsequently sequenced. DNA sequences were determined using an automated DNA sequencer (Applied Biosystems, Model 377), and analyzed with the Omiga 2.0 sequence analysis software package (Oxford Molecular Group).

**Statistics:** Fisher’s exact test was used to measure the significant differences in the percentage of colonized chickens at each sampling point between groups (Statistical Analysis System, SAS Institute Inc.). One-way analysis of variance followed by an LSD (Least Significant Difference) test was used to calculate the significant differences in shedding level (log-transformed) and ELISA OD values at each sampling point among groups. A *p* value of less than or equal to 0.05 was considered significant.

### 3.4 RESULTS

*Campylobacter colonization in commercial broilers.* The 3-day old commercial broiler chicks inoculated with a low dose (3X10^2 CFU/bird) of strain S3B (Exp. I, Group 1) did not shed the organism until day 7 post-challenge (Table 3.3). Thereafter,
the proportion of colonized chickens abruptly reached 100 %, and stayed at this level until the end of the experiment on day 28 post-challenge. The numbers of *C. jejuni* cells shed in feces of colonized chickens (log$_{10}$ CFU/g feces) ranged between approximately 5 and 7.5 log-transformed units (results not shown). In contrast to the 3-day old broilers, *Campylobacter* shedding was detected on day 3 post-inoculation in 90 % of the 21-day old birds following challenge with the same dose (3X10$^2$ CFU/bird) of the same strain (Table 3.3, Exp. I, Group 2). The percentage of colonized chickens decreased substantially three weeks after inoculation in the chickens challenged at 21 days of age (Group 2). However, the proportion of colonized chickens remained 100% until the end of the experiment in the chicks challenged at 3-days of age (Group 1).

In experiment II using 21190 as the challenging strain, infection of 3-day old chicks with a low dose (3X10$^2$ CFU/bird) of 21190 did not result in colonization of any of the birds throughout the experiment (Table 3.3, Exp. II, Group 1). However, 21-day old birds (Group 2) became colonized as early as day 3 post-challenge following inoculation with the same dose (3X10$^2$ CFU/bird) of strain 21190. The numbers of *C. jejuni* cells shed in feces of colonized chickens (log$_{10}$ CFU/g feces) ranged between approximately 4 and 5.8 log-transformed units (results not shown). Similar to what was observed in experiment I, the proportion of colonized birds in the group infected at the age of 21 days decreased over time. In fact, none shed the organism in the feces after day 15 post-challenge.
Together, these observations indicated that, although there was strain-associated variability in *Campylobacter* colonization of chickens, older chickens (3-weeks old) were consistently more susceptible to *C. jejuni* infection than younger ones (3-day old). This age-related disparity in susceptibility to *Campylobacter* colonization might be due to the difference in antibody levels, because the 21-day old birds had no antibodies to *Campylobacter*, while 3-day old chicks had high levels of MAB against this organism (Table 3.1). To confirm the role of MAB in protecting from *Campylobacter* colonization, a defined challenge system with SPF chickens was used.

*Campylobacter colonization in SPF White Leghorn chickens.* The effect of MAB on *Campylobacter* colonization was further assessed using 3-day old MAB+ or MAB− SPF birds (Table 3.2). The chicks were challenged with either strain S3B, which was used to infect the laying hens, to examine the homologous protection, or strain 21190 to assess the heterologous protection. As shown in Table 3.4, following infection with a relatively low dose (5×10^3 CFU/bird) of S3B, the percentages of colonization in the MAB+ birds (Group 1) were consistently lower than those in the MAB− birds (Group 3) on days 2, 4, 6, 8, and 10 post-challenge (Table 3.4, Exp. I), although the differences were not statistically significant. When challenged with a higher dose (5×10^5 CFU/bird) of the same strain (S3B), a similar trend was observed with significant differences between the MAB+ group (Group 2) and the MAB− group (Group 4) on days 2, and 4 post-challenge (Table 3.4, Exp. I). A 100% colonization rate in all groups was reached on day 12 post-inoculation and then stayed at the same level until the end of experiment on day 28 post-challenge. The mean number of organisms (log_{10} CFU/g feces) shed in
feces of colonized birds ranged between 4.9 and 6.3. In contrast to the colonization percentage, there were no significant differences (data not shown) in the mean number of *C. jejuni* organisms shed in the feces of colonized birds in groups infected with the same dose of the organism regardless of the status of *Campylobacter*-specific MAB.

When 3-day-old MAB\(^+\) and MAB\(^-\) chickens were infected with a relatively low dose (2\(\times\)10\(^4\) CFU/bird) of strain 21190, the percentages of birds colonized in MAB\(^+\) group (Group 1) were significantly lower than those seen in MAB\(^-\) group (Group 3) on days 2, 4, and 6 post-challenge (Table 3.4, Exp. II). Notably, on day 2 post-challenge, none of the chicks were colonized in the MAB\(^+\) group, although 5 (45\%) of 11 birds in MAB\(^-\) group excreted the organism in feces. Slightly lower rates of colonization in MAB\(^+\) birds were also noticed on days 8 and 10 post-challenge than those observed in MAB\(^-\) birds. Following infection with a high dose (2\(\times\)10\(^6\) CFU/bird) of strain 21190, significantly lower rates of colonization occurred on days 2 and 4 post-challenge in the group with MAB\(^+\) birds (Group 2) than the group with MAB\(^-\) birds (Group 4). Also, the colonization rate in the MAB\(^+\) group on day 6 post-challenge was considerably less, but not statistically significant, than that in the MAB\(^-\) group (Table 3.4, Exp. II). By day 12 post-challenge, a 100\% colonization rate was observed in all groups. The mean numbers of *C. jejuni* organisms excreted in feces (log\(_{10}\) CFU/g feces) of the colonized chickens were between 5.4 and 6.7 (results not shown). Similar to the challenge with strain S3B, there were no significant differences at a given sampling point in the mean numbers of *C. jejuni* shed in feces of colonized birds between groups challenged with the same dose of 21190 regardless of the MAB status of the birds. Together, the results from the challenge
experiments using SPF chickens further confirmed that *Campylobacter*-specific MAB was associated with the partial protection against *C. jejuni* colonization in chickens.

**Age-related difference in active immune response to *Campylobacter* colonization in broiler chickens.** To determine the development of active antibody responses elicited by *Campylobacter* colonization in broiler chickens at different ages, serum IgG antibody responses were measured following experimental infection of broilers. Comparison of serum IgG antibody responses in chickens infected with S3B on day 3 or 21 of age indicated that active immune response to *Campylobacter* occurred sooner and at significantly higher levels in older chickens than in younger ones (Table 3.3, Exp. I). A noticeable level of active IgG response was not detected until the fourth week following the infection of 3-day old chicks, whereas the chickens inoculated at 21 days of age mounted a marked response to the infection as early as one week after the challenge. These results indicated that dynamics of active antibody response in broiler chickens to *Campylobacter* colonization differs with the age of the birds at challenge. In chickens challenged on day 21 of age, the decreases in the percentage of birds colonized with either *Campylobacter* strain seemed to be correlated with increasing titers of anti-*Campylobacter* serum IgG, suggesting a potential role of active antibody response in reducing colonization of chickens by *C. jejuni*.

**Effect of MAB on active immune responses to *Campylobacter* colonization in SPF White Leghorns.** To determine if *Campylobacter* MAB affects the development of an active immune response to *Campylobacter* colonization, the presence of
Campylobacter-specific antibodies in serum was determined before and after challenge of 3-day old White Leghorn chickens (Table 3.5). As expected, 2-day old chicks from eggs of Campylobacter-infected hens (Groups 1 and 2) had high levels of maternally-acquired IgG antibodies prior to the challenge, whereas chicks from eggs of Campylobacter-free SPF hens (Groups 3 and 4) were negative for Campylobacter antibody. The level of maternal IgG antibody decreased gradually beginning on days 6, 14 and 21 post-challenge. The maternal transfer of IgM and IgA antibodies from hens to the progeny was negligible, although very low levels of maternal IgA were detected in chicks derived from Campylobacter-infected parent flock (Groups 1 and 2). With respect to the production of an active humoral immune response to C. jejuni colonization, only weak IgM and IgA responses were elicited during the first two weeks post-infection, followed by a gradual increase in the antibody levels during the next two weeks. A small active IgG antibody response was observed only after 4 weeks post-infection, which is consistent with the slow development of active IgG antibody responses in experimentally infected 3-day old broiler chickens (Table 3.3, Exp. I, Group 1). Despite some variations in the IgM and IgA ODs in active antibody responses to C. jejuni colonization, the levels of antibodies at a given sampling point for these immunoglobulin isotypes did not differ significantly regardless of the MAB status of the birds at challenge (Table 3.5). The dynamics of active immune responses were similar in birds challenged with strain S3B or 21190. These findings demonstrated that following Campylobacter infection, the IgA and IgM antibody responses occurred earlier and at much higher levels than the IgG responses, and the presence of MAB did not significantly affect the development of an active serum IgG response to C. jejuni.
In vitro bactericidal effect of MAB on Campylobacter. When heat-inactivated sera from 2-day old broilers (with high MAB level) was incubated with *C. jejuni* strain S3B in the presence of an exogenous complement source, a 22 % reduction in CFU counts was observed. However, the same sera had a substantially higher rate (46 %) of killing of strain 21190 (Table 3.6). On the contrary, sera obtained from MAB+ White Leghorn chicks, which were the progeny of S3B-infected hens, killed 100 % of the homologous strain S3B in the presence of complement, but had no effect on the heterologous strain 21190 (Table 3.6). These results indicated that chicken MAB was highly effective and strain-specific in complement mediated killing of homologous Campylobacter strains in vitro. These result also showed that specific maternal antibodies from different sources had varying in vitro killing effects on different *C. jejuni* strains. Sera from MAB- White Leghorn chicks, however, did not result in any reduction in CFU counts of either strain, further indicating the specificity of complement-mediated killing. Although the sera from the MAB+ SPF chickens had no bactericidal effect on strain 21190, the chickens were partially protected against the colonization by 21190 (Table 3.4), suggesting that antibody-dependent complement-mediated killing is not the only mechanism for the protective immunity.

Genotyping of Campylobacter isolates from the chickens. To confirm that the isolates recovered from the experimental chickens were derived from the inoculated S3B or 21190 strains, representative Campylobacter isolates obtained at different sampling points were analyzed by sequencing of the cmp gene, which is diverse among different strains (41). With respect to each challenge experiment involving different *C. jejuni*
strains, *Campylobacter* isolates recovered from the chickens showed a *cmp* gene sequence indistinguishable from the challenge strain (data not shown). This result indicated that the most likely source of the recovered *Campylobacter* was from the inoculum used in the experiments and not extraneous sources.

### 3.5 DISCUSSION

Results from the present study demonstrated that colonization of chickens with *C. jejuni* varies with different doses and strains, and the age of the host at the time of oral challenge. More importantly, our results indicate for the first time that *Campylobacter*-specific MAB in young chickens confers partial protection against colonization upon challenge with both homologous and heterologous *C. jejuni* strains. This conclusion is supported by two complementary observations. First, older broiler chickens (21-day old), negative for *Campylobacter*-specific antibody, were much more susceptible to colonization with *C. jejuni* than were 3-day-old young broilers that had high levels of *Campylobacter* MAB (Table 3.3). Second, 3-day-old SPF White Leghorn chicks with high levels of *Campylobacter*-specific MAB showed significantly less colonization during the first week after challenge as compared with 3-day-old SPF chicks without *Campylobacter*-specific MAB (Table 3.4). The demonstrated protectiveness of MAB and its high prevalence in young chickens as shown in a previous study (31) suggest that *Campylobacter* MAB is a contributing factor for the lack of *Campylobacter* colonization in young broiler birds under commercial production. The ability of MAB to reduce *Campylobacter* colonization provides evidence that immunization-based approaches may
be effective as a means to initially control *Campylobacter* in broiler flocks at the farm level.

Antibodies present in the systemic circulation of breeder chickens can be readily transferred to the egg yolk. During hatching, the yolk immunoglobulins are absorbed into the embryonic circulation as MAB. The transferred maternal antibodies are mainly of IgG class, and minimal passage of IgA and IgM from breeders to progenies occurs (20, 21, 28). In the present study, we observed similar findings regarding the maternal transfer of *Campylobacter* antibodies. The predominant immunoglobulin detected in 2-day old chicks hatched from eggs from *Campylobacter*-infected hens was IgG, although maternal transfer of small amounts of IgA antibody also occurred (Table 3.5). Chicks hatched from eggs from *Campylobacter*-free SPF hens lacked detectable levels of specific *Campylobacter* antibodies. Since the transudation of circulating maternal IgG antibody to the intestine of chicks during the first week of life is high (20, 21, 34), the measurement of the serum IgG titers in young broilers provides a reasonable estimation on the level of MAB in the intestinal tract of the birds during the first week of life.

Maternal antibodies in young birds are known to be protective against infectious agents including viruses, bacteria, and parasites that colonize the intestinal epithelium of poultry during the early stage of their life (14, 15, 34, 36). Studies performed with mammalian hosts including humans, monkeys, mice, ferrets, and rabbits indicated that passively acquired *Campylobacter*-specific MAB or active anti-*Campylobacter* immunity induced by vaccination or natural exposure were protective against subsequent infection with *C. jejuni* (2, 4, 6, 9, 27, 29, 30). In humans, breast-feeding of newborns was associated with decreased numbers of cases and duration of diarrhea caused by *C. jejuni*,
indicating the protective effect of MAB (29). Also, intraperitoneal vaccination of female mice with a killed whole-cell *C. jejuni* resulted in transfer of MAB to the pups, which were protected from an oral challenge on days 4 to 6 after birth with the homologous strain (9). The results obtained in this study indicated a partial protection of young birds by *Campylobacter*-specific MAB, although this protection was limited and did not prevent the birds from eventual colonization by *C. jejuni*. Collectively, these findings strongly indicate that specific MABs are able to retard *C. jejuni* colonization in various animal hosts. The mechanisms by which MAB confers protection against *Campylobacter* colonization are unknown. It is possible that the MAB in the intestinal tract of young chickens during the first week of age may block bacterial adhesins on the surface of *Campylobacter*, agglutinate the organism, interfere with the chemotaxis toward receptor sites, or kill the organism via complement-mediated killing. We found that *Campylobacter*-specific MAB was also effective against challenge with the heterologous *Campylobacter* strain 21190, to which the breeders were not exposed. This observation suggests that *Campylobacter* MAB naturally occurring in young broiler chickens on farms may confer a broad protection against different strains of *C. jejuni*.

The development of active humoral immune responses following *C. jejuni* infection of 3-day old chickens was also monitored (Tables 3.3 and 3.5). A slowly-progressive humoral immune response was elicited against *C. jejuni* colonization, in which IgM and IgA responses preceded those of IgG. Despite some variations, the level of an immunoglobulin isotype did not differ significantly between groups at a given sampling point regardless of the MAB status of the birds and the challenge dose (Table 3.5). These observations suggested that the presence of high levels of MAB in young
chickens did not interfere with the development of a humoral immune response to *Campylobacter* colonization, in agreement with a previous observation (40). However, the effect of MAB on the development of active local immune responses in the intestinal tract was not investigated in this study, and remains to be determined. The lack of interference by MAB on the development of an active systemic immune response to *Campylobacter* may have positive implications for vaccination of young chickens, which needs to be explored in future studies. Our observation on the kinetics of active immune responses to *C. jejuni* colonization was consistent with previous findings reported by other investigators (7, 24, 26), in which circulating IgM and IgA antibodies occurred much earlier than the IgG response in the chickens infected with *C. jejuni*.

After inoculation with *Campylobacter*, older (21-day old) broilers mounted a substantially higher level of humoral immune response within a short period than did the young broilers (3-day old) (Table 3.3). Widders *et al.* (40) also reported the production of poor immune responses in day-old chicks immunized with flagellin protein, whereas 24-day old chickens produced significantly higher levels of serum IgG antibodies specific for the flagellin of *C. jejuni*. The differences in the levels of active immune responses in different age groups of chickens are likely to result from the fact that the immune system of newly hatched chicks is immature and only partially developed (18, 32). Interestingly, the percentage of colonized chickens dropped dramatically 2-4 weeks after challenge of 21-day old broilers, although such marked reductions were not noticed following infection of 3-day old chicks (Table 3.3). Others also noted the shorter duration of shedding of *Campylobacter* in both experimentally and naturally infected older chickens as compared with younger birds (19, 22). The reduction in colonization was accompanied
with the formation of *Campylobacter* IgG antibodies in chickens infected with S3B (Table 3.3). Similar observations were also found in a previous study, which showed that the decrease in the number of viable *Campylobacter* organisms in feces was associated with the development of specific serum agglutinin antibodies in experimentally infected chickens at the age of 3-months, but not in chickens infected at 3-days or 5-weeks of age (19). However, a correlation between the clearance of *Campylobacter* and the development of specific IgG was not apparent in the 21-day old chickens inoculated with strain 21190, since the birds stopped shedding of the organism before a substantial IgG response was observed (Table 3.3). Nonetheless, this does not exclude the possibility that early IgA or IgM responses contributed to the clearance of *Campylobacter* from the chickens challenged at 21-day of age with 21190. The role and nature of active immune responses in protecting chickens from *Campylobacter* colonization remains to be determined.

Previously, we showed that *Campylobacter*-specific MAB was active in killing *C. jejuni* in a strain-specific manner (31). In this study, MAB from commercial broilers, whose parents are likely to be infected with multiple strains of *C. jejuni* under natural conditions, were effective in the killing of strain 21190, but less effective in the killing of strain S3B (Table 3.6). In contrast to the commercial broiler serum, MAB in 2-day old White Leghorn chicks, whose SPF parents were artificially infected with S3B, completely killed the homologous strain (S3B) in the presence of complement, but had no effect on the heterologous strain 21190 (Table 3.6). These results further indicate the strain-dependent specificity of complement-fixing MAB. It is likely that the *Campylobacter* surface antigens inducing complement-fixing antibodies are antigenically
variable among different strains, resulting in variation in the MAB-dependent complement-mediated killing of different isolates. Despite the great differences in susceptibility to the \textit{in vitro} bactericidal effects of MAB, both S3B and 21190 showed similar colonization characteristics in 3-day old White Leghorns (Table 3.4). This suggests that the complement-fixing ability of MAB is not the only factor contributing to the protection. Thus, other unknown mechanisms are also likely responsible for the partial protection against \textit{Campylobacter} colonization in young chickens conferred by MAB.

3.6 ACKNOWLEDGMENTS

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3.7 REFERENCES


<table>
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<tr>
<th>Exp.</th>
<th>Group</th>
<th># birds</th>
<th>age&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Challenge dose (CFU/bird)</th>
<th>Serum MAB&lt;sup&gt;d&lt;/sup&gt; level at challenge</th>
<th>Sampling scheme at days after challenge&lt;sup&gt;e&lt;/sup&gt;</th>
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<sup>a</sup>Challenged with strain S3B  
<sup>b</sup>Challenged with strain 21190  
<sup>c</sup>Days of age at challenge  
<sup>d</sup>Mean ELISA OD ± SD  
<sup>e</sup>“S” represents collection of cloacal swabs for isolation of *Campylobacter* from feces. “B” indicates collection of blood samples for testing antibodies.

**Table 3.1** Experimental design of challenge studies using commercial broiler chickens
<table>
<thead>
<tr>
<th>Exp. Group</th>
<th># bird</th>
<th>Age</th>
<th>Challenge dose (CFU/bird)</th>
<th>Serum MAB level at challenge</th>
<th>Sampling scheme at days after challenge</th>
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\( ^a \) Challenged with strain S3B
\( ^b \) Challenged with strain 21190
\( ^c \) Days of age at challenge
\( ^d \) Mean ELISA OD ± SD
\( ^e \) “S” represents collection of cloacal swabs for isolation of *Campylobacter* from feces. “B” indicates collection of blood samples for testing antibodies. NT: Not tested

**Table 3.2** Design of challenge experiments using SPF White Leghorn chickens
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<th>% colonization on day after challenge</th>
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¹Experiment and group numbers correspond to those in Table 1.
²Proportions in the same column of each experiment with different superscript letters differ significantly (Fisher’s exact test).
³Mean ELISA OD for 7 to 10 serum samples.
⁴Means in the same column with different superscript letter differ significantly (one-way ANOVA).
⁵Not tested.

Table 3.3 Colonization percentages and serum IgG levels in broiler chickens following challenge with *C. jejuni* strains S3B or 21190
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<sup>1</sup> Experiment and group numbers correspond to the ones in Table 2.
<sup>2</sup> Proportions in the same column of each experiment with different superscript letters differ significantly (Fisher’s exact test; values with no significant difference are left unmarked).
<sup>3</sup> Not tested

**Table 3.4** Colonization percentages in SPF chickens following challenge with *C. jejuni* strains S3B or 21190
Table 3.5 Levels of *Campylobacter*-specific serum antibodies before and after challenging of 3-day-old SPF chickens with two *C. jejuni* strains\(^a\)
<table>
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<th>Ab Class and Group</th>
<th>Experiment I (S3B-inoculated) Serum ELISA OD after challenge on day&lt;sup&gt;b&lt;/sup&gt;:</th>
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<tr>
<td>1</td>
<td>0.086</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>(0.017)</td>
<td>(0.025)</td>
</tr>
<tr>
<td>2</td>
<td>0.082</td>
<td>0.142</td>
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<tr>
<td></td>
<td>(0.012)</td>
<td>(0.014)</td>
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<tr>
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<td>0.075</td>
<td>0.139</td>
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<tr>
<td></td>
<td>(0.015)</td>
<td>(0.039)</td>
</tr>
<tr>
<td>4</td>
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<td>0.111</td>
</tr>
<tr>
<td></td>
<td>(0.015)</td>
<td>(0.011)</td>
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<tr>
<td><strong>IgA</strong></td>
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</tr>
<tr>
<td>1</td>
<td>0.199&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>(0.027)</td>
<td>(0.035)</td>
</tr>
<tr>
<td>2</td>
<td>0.211&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td>(0.032)</td>
<td>(0.014)</td>
</tr>
<tr>
<td>3</td>
<td>0.047&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>(0.035)</td>
<td>(0.039)</td>
</tr>
<tr>
<td>4</td>
<td>0.046&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.025)</td>
<td>(0.011)</td>
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<td><strong>IgG</strong></td>
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<tr>
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<td>0.783&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>(0.074)</td>
<td>(0.068)</td>
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<tr>
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<td>0.733&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.056)</td>
<td>(0.065)</td>
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<tr>
<td>3</td>
<td>0.201&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.138&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.017)</td>
<td>(0.015)</td>
</tr>
<tr>
<td>4</td>
<td>0.206&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.118&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.026)</td>
<td>(0.014)</td>
</tr>
</tbody>
</table>
Experiment and groups numbers correspond to the ones in Table 2.

Mean OD value of 5-10 serum samples. Numbers in parenthesis represent standard errors. Means in the same column of each antibody-class with different superscript letters differ significantly (one-way ANOVA; values with no significant difference are left unmarked).

Table 3.5 Levels of *Campylobacter*-specific serum antibodies before and after challenging of 3-day-old SPF chickens with two *C. jejuni* strains$^a$
<table>
<thead>
<tr>
<th>Strains</th>
<th>Mean % reduction in CFU by maternal antibodies&lt;sup&gt;1&lt;/sup&gt; (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPF&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>S3B</td>
<td>100 (0)</td>
</tr>
<tr>
<td>21190</td>
<td>-1.5 (10.5)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are means of four independent experiments.
<sup>2</sup> Pooled sera from 2-day-old *Campylobacter*-free MAB<sup>+</sup> SPF White Leghorns whose hens were experimentally-infected with S3B.
<sup>3</sup> Pooled sera from 2-day old *Campylobacter*-free MAB<sup>-</sup> SPF White Leghorns whose hens were *Campylobacter*-free.
<sup>4</sup> Pooled sera from 2-day old *Campylobacter*-free commercial broilers having high levels of *Campylobacter* MAB.

**Table 3.6** Bactericidal activity of *Campylobacter*-specific maternal antibody on *C. jejuni* strains
CHAPTER 4

DETECTION AND SURVIVAL OF CAMPYLOBACTER IN CHICKEN EGGS

4.1 ABSTRACT

Campylobacter jejuni, a foodborne human pathogen, is widespread in poultry; however, the sources of infection and modes of transmission of this organism on chicken farms are not well understood. The objective of this study was to determine if vertical transmission of C. jejuni occurs via eggs. Using a temperature differential method, it was shown that Campylobacter was either unable to penetrate the eggshell or did not survive up to 48 h inside the incubating eggs. When C. jejuni was inoculated into the egg yolk and the eggs were stored at 18°C, the organism was able to survive for up to 14 days. However, viability of C. jejuni was dramatically reduced when injected into the albumen or the air sac. When freshly-laid eggs from Campylobacter-inoculated SPF layers were tested, C. jejuni-contamination was detected in 3 of 65 pooled whole eggs (5-10 eggs for each pool) via culture and PCR. However, the organism was not detected from any of the 800 eggs (80 pools), collected from the same SPF flock, but kept at 18°C for 7 days before testing. Likewise, Campylobacter was not recovered from any of 500 fresh eggs obtained from commercial broiler breeder flocks that were naturally colonized by Campylobacter. Also, none of the 1,000 eggs from broiler breeders obtained from a
commercial hatchery were positive for *Campylobacter*. These results suggest that vertical transmission of *C. jejuni* through the egg is probably a rare event and does not play a major role in the introduction of *Campylobacter* to chicken flocks. Thus, control of *Campylobacter* transmission to chicken flocks should focus on sources of infection that are not related to eggs.

**4.2 INTRODUCTION**

*Campylobacter jejuni* is the leading cause of bacterial foodborne illnesses in the United States and other developed countries (2, 19), and also is frequently associated with diarrhea in patients <6 months of age in developing countries (13). An estimated 2.1 to 2.5 million cases of human campylobacteriosis, characterized by watery and/or bloody diarrhea, occur annually in the United States, exceeding the cases of salmonellosis (19). In addition, *C. jejuni* is the most frequent antecedent of Guillain-Barre Syndrome (GBS), an acute demyelinating disorder of peripheral nerves, which may lead to respiratory muscle compromise and death (31, 42). The vast majority of human campylobacteriosis cases occur sporadically, and primarily result from consumption of undercooked poultry or other foods cross-contaminated with raw poultry meat during food preparation (14, 19, 23). However, others risk factors besides poultry, such as contact with house pets, or consumption of raw milk, untreated water, and undercooked beef or pork, have also been linked to human infections (14, 33). Since poultry are considered a major reservoir for human campylobacteriosis, reduction or elimination of poultry contamination with *C. jejuni* would greatly reduce the risk of *Campylobacter* for public health. To achieve this goal, it is essential to understand the ecology of *Campylobacter* in the poultry production
systems so that effective intervention strategies can be designed and implemented at the preharvest stage.

Although numerous farm-based studies have been conducted in the past decades, the sources of flock infection, modes of transmission, and the host and environmental factors affecting the spread of *Campylobacter* on poultry farms are still poorly understood (35). There has been a major debate on whether vertical or horizontal transmission is responsible for the introduction of *Campylobacter* into chicken flocks. For years, the prevailing theory has been that horizontal transmission from the environment is the major source of *C. jejuni* infection for broiler flocks, and that vertical (egg-borne) transmission is unlikely. Potential sources of flock infection include used litter, untreated drinking water, other farm animals, domestic pets, wildlife, house flies, insects, farm equipment and workers, and transport vehicles (5, 18, 24, 26, 40). However, none of these suspected sources has been conclusively identified as the formal source of infection for broilers farms. In many cases, it was difficult to determine which events (the flock infection or environmental contamination) occurred first since no study plan was included to monitor the direction of *Campylobacter* transmission.

However, some circumstantial evidence suggests that vertical transmission of *C. jejuni* from breeder flocks to the broiler farms via the egg may occur. Earlier studies showed that, *C. jejuni* could be isolated from outer (17) and inner (38) surfaces of eggs laid by naturally infected commercial layers or broiler breeders. Shane *et al.* (37) isolated the organism from both interior surface of egg shell and egg contents after swabbing feces containing *C. jejuni* onto the egg surface. Following experimental infection of eggs with *C. jejuni* by either temperature differential method (10) or inoculation of egg
albumen via direct injection (38), the organism was recovered from both the contents of unhatched eggs and from the newly hatched chicks. Recent investigations using sensitive molecular detection methods demonstrated the presence of *Campylobacter* DNA in embryos and newly hatched chicks (8, 9), and in hatcheries (22). Furthermore, *C. jejuni* has been isolated from the reproductive tract of healthy laying and broiler breeder hens (7, 24) and from semen of commercial broiler breeder roosters (16). The same *C. jejuni* genotypes have been identified from breeder flocks and their progeny flocks (15, 34), suggesting that breeder hens might be the source of *Campylobacter* infection for progeny flocks.

Despite these observations, vertical transmission of *C. jejuni* is still questionable because live *Campylobacter* have not been detected in the contents of commercial breeder eggs, young hatchlings, or hatcheries under natural conditions (4, 17, 22, 26, 37, 38). Therefore, the exact role of vertical transmission in introducing *Campylobacter* to broiler flocks remains unclear. The purpose of this study was to evaluate the importance of vertical transmission as a source flock infection by *C. jejuni* on broiler farms. Toward this end, we determined the ability of *C. jejuni* to penetrate eggshells and to survive in different compartments of the egg. In addition, the prevalence of the organism in eggs obtained from multiple broiler flocks, a hatchery, and laboratory-infected hens was determined using both culture and PCR methods.
4.3 MATERIAL AND METHODS

Methods for detection of Campylobacter in eggs: To develop a sensitive detection method that would recover low numbers of Campylobacter from eggs, specific pathogen-free (SPF) eggs free of Campylobacter were artificially infected with relatively low doses of C. jejuni and various detection techniques were tested to assess the sensitivity of each method. Eggs were obtained from Campylobacter-negative SPF White Leghorn layers maintained at our department’s poultry facilities. Outer surface of the egg shell was disinfected with 70% ethanol and air-dried. Contents and shell membranes of 10 eggs were aseptically separated and pooled separately. Approximately 10 ml of pooled egg content or 5 g of pooled shell membranes were then mixed with 90 or 45 ml, respectively, of Mueller-Hinton (MH) broth containing Campylobacter growth supplements (Oxoid) in sterile bottles. Each mixture was spiked with varying numbers (Table 1) of C. jejuni strain S3B (a chicken isolate), and subjected to an enrichment step for 48 h at 42°C under microaerophilic conditions produced by CampyPack Plus gas-generating envelopes (BBL Microbiology System, Cockeysville, MD). One hundred µl enrichment culture was then spread onto MH agar plates containing Campylobacter-specific growth supplements (Oxoid), and incubated microaerobically as described above for Campylobacter isolation.

Besides direct plating of enrichment cultures onto MH agar, the enrichment cultures were also subjected to a density gradient centrifugation step to separate egg materials from bacteria using gradient medium Percoll® (Pharmacia Biotech, Sweden) as described elsewhere (39, 41). This method is based on the fact that bacteria have higher buoyant densities than most food homogenates, and thus can be separated from foods in a
gradient medium such as Percoll®. Briefly, a standard isotonic medium (SIM) was made by adding 100 mg peptone and 850 mg NaCl to 100 ml of Percoll®. The bacterial cells in the enriched egg samples were separated from the enrichment mixtures by carefully layering 0.9 ml of sample over 0.6 ml of 40% SIM in peptone water (8.5 g NaCl and 1.0 g peptone in 1 L Millipore water) and centrifuging at 16,000 X g for 1 min. The supernatant was gently removed down to a volume of 0.1 ml. The remaining 0.1 ml dense gradient medium in the bottom of the tube was added to 1 ml saline solution and mixed gently. The tube was centrifuged again at 9,500 X g for 5 min, after which the supernatant was removed down to a final volume of approximately 100 µl. This volume was then spread onto MH agar plates for isolation of the bacterium.

For polymerase chain-reaction (PCR) detection of *Campylobacter* from eggs, enrichment cultures were also subjected to a density gradient centrifugation using Percoll® as described above to remove egg materials and PCR inhibitors. The final volume of approximately 100 µl of suspension obtained after the second centrifugation was used to isolate bacterial DNA using Dneasy Tissue Kit (Qiagen). A PCR assay targeted to amplify a region of 16S rRNA gene specific for *C. jejuni* and *C. coli* was employed as described previously (27). The forward primer (CCCJ609F; 5’-AAT CTA ATG GCT TAA CCA TTA-3’) and the reverse primer (CCCJ1442R; 5’-GTA ACT AGT TTA GTA TTC CGG-3’) were designed from the published sequences (27). Amplification was performed in a 100-µl reaction volume containing 10 µl of sample DNA or 100 ng positive control DNA, 0.5 µM of each primer, 1.5 mM MgCl₂, 200 µM of each oligonucleotide, and 2.5 U of Taq DNA polymerase (Progmega). The PCR amplification was performed with a PC-200 thermocycler (GeneAmp® PCR System
The reactions were subjected to an initial denaturation for 2 min at 95°C, followed by 30 amplification cycles, each consisting of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. A final primer extension at 72°C for 10 min was included for each reaction.

**Experimental egg penetration studies:** The ability of *C. jejuni* to penetrate the eggshell as well as outer and inner eggshell membranes was investigated using a temperature-differential process as described previously with minor modifications (10). This method works based on the assumption that a warm egg contracts while cooling, and the resultant negative pressure can draw bacteria into/through the eggshell. Briefly, a total of 50 fertile broiler eggs were obtained from a local commercial hatchery, and equilibrated at 42°C in an incubator for 3 h. A stationary-phase culture of *C. jejuni* strain S3B was added to a freshly-collected fecal suspension (in MH broth) obtained from *Campylobacter*-free SPF hens. The final amount of the organism in this suspension was 2 X 10^5 CFU ml^-1 as determined by viable CFU counting. The eggs that were held at 42°C for 3 h were placed in the fecal suspension inoculated with *C. jejuni*, and kept there for 30 min at 22°C. The eggs were then withdrawn, rinsed three times with sterile water, and allowed to air dry for 1 h at room temperature before being placed in an incubator for hatching. The isolation of *C. jejuni* from eggs was done at days 2, 6, 10, and 15 of incubation. At each time of examination, five eggs were removed, and each whole-egg was mixed thoroughly using a blender. Twenty ml blended egg was then added to 200 ml of MH broth containing *Campylobacter* growth supplements (Oxoid). Following the enrichment step for 2 days under microaerophilic atmosphere at 42°C, isolation of
Campylobacter colonies was attempted using the culture methods as described above. Also, the 17 hatched chicks were placed on a wire-floored cage in a steam-cleaned and formaldehyde-fumigated isolation room, and provided with Campylobacter-free water and feed \textit{ad libitum}. Cloacal swabs were taken right after placement, and then once per week for the next 6-week period to isolate \textit{Campylobacter} as described previously (28).

**Survival of \textit{Campylobacter} in experimentally infected eggs:** The viability of \textit{C. jejuni} inoculated into the egg yolk, albumen, and air sac was examined as follows. For the egg yolk inoculation, a total of 50 eggs, 25 of which from a commercial broiler hatchery and 25 of which from \textit{Campylobacter}-free SPF hens, were surface disinfected with 70% ethanol and air-dried. Using a sterile needle, 0.1 ml aliquots (ca, 4.5 $\times$ 10$^2$ CFU for each egg) of stationary-phase culture of \textit{C. jejuni} strain S3B were injected into the yolk. The injection hole was sealed with tape, and the eggs were kept at 18°C. At different time points, i.e., 2, 5, 8, 11, and 14 days after inoculation, 5 egg contents from each group were aseptically collected and separately tested for \textit{Campylobacter} using both direct plating or the enrichment culture as described above. For inoculation of the egg albumen and the air sac, eggs obtained from \textit{Campylobacter}-free SPF hens were used (25 eggs for each group). The procedure of egg inoculation, storage conditions and isolation methods were similar to those of egg yolk injection, except that the inoculation was into the albumen or air sac. The inoculum was approximately 7 $\times$ 10$^2$ CFU for each egg. When testing the viability of organism in the air sac following storage at 18°C, a whole-egg (instead of just the contents) was first mixed using a blender and, then cultured for isolation of \textit{Campylobacter} via the enrichment method as described above.
Detection of *C. jejuni* in eggs laid by experimentally-infected layers: To determine the extent of contamination by *Campylobacter*, eggs laid by *C. jejuni*-infected hens were surveyed. An SPF layer flock free of *Campylobacter* was maintained in the laboratory animal facilities of our department. The absence of colonization of these birds (15 pullets and 4 cockerels) with *Campylobacter* was confirmed by culturing of cloacal swabs as described previously (28). After they started laying eggs, hens were orally inoculated with *C. jejuni* strain S3B (5 X 10^7 CFU for each bird) at the age of 22-weeks. Following establishment of *Campylobacter*-colonization (as determined by cloacal swabbing) in the hens, eggs were collected daily over a period of a year. Hens were re-inoculated several times during the egg collection period with the same strain of *C. jejuni* via drinking water to ensure that they would constantly shed *Campylobacter* in their feces. Fresh fecal droppings were cultured weekly to isolate *Campylobacter*. Of the eggs collected throughout the study period, approximately 500 were examined on the day of collection, while about 800 were kept at 18°C for 7 days before bacteriological examination. In both cases, eggs were not washed or disinfected before examination. To isolate *Campylobacter*, a pool of 5-10 whole eggs was mixed using a blender. Twenty ml of the blended eggs was then added to 200 ml of MH broth containing *Campylobacter* growth supplements (Oxoid). Following an enrichment step first, detection of *Campylobacter* was attempted using both the culture and the PCR methods as mentioned above. To determine whether the source of *C. jejuni* isolated from eggs was the *Campylobacter*-infected laying hens, isolates recovered from the eggs and those from the feces of layers were compared by sequencing of the *cmp* gene (encoding a major outer
membrane protein), which is divergent among different *Campylobacter* strains as described previously (43).

Besides testing of eggs laid by *Campylobacter*-infected layers, we also investigated the presence of *C. jejuni* in the young chicks of layers. In two instances, eggs (70 eggs each time) were placed in an incubator for hatching when the shedding of organism in feces of hens was at high levels (ca, log$_{10}$ 6.5 CFU g$^{-1}$ of feces). The newly hatched chicks (a total of 95) were tested via cloacal swabbing on day 2 of age to determine whether they had *Campylobacter* as described previously (28).

**Survey of commercial broiler breeders and eggs:** A local broiler breeder operation consisting of flocks spread out at different locations was chosen to survey the level of *Campylobacter* contamination in eggs laid by naturally infected hens. Five commercial breeder flocks at the age of 25 to 50 weeks were investigated during the Fall of 2002. Each flock was located at different premises, with a minimum distance of 20 miles between them. Fifty fresh fecal droppings were collected from each of the flocks and cultured within a few hours of collection via direct plating as described previously (28). Colonies with *Campylobacter*-like morphology were further identified using a PCR specific for *C. jejuni* and *Camp. coli* (27). Within 2-weeks of collection of fecal samples, 100 commercial eggs were obtained from each breeder flock that shed *Campylobacter* (a total of 500 eggs were tested). These eggs were less than 2 days old, and were not subjected to any washing or disinfection procedure. Fecal contamination was apparent on the surface of many eggs. To isolate *Campylobacter* from eggs, a pool of 10 whole eggs (shell and contents) was mixed together using a blender. Twenty ml of the blended eggs
was then added to 200 ml of MH broth containing *Campylobacter* growth supplements (Oxoid). Following an enrichment step, detection of the organism was attempted by culture and PCR methods as aforementioned.

**Survey of eggs from a commercial hatchery for broilers:** To determine the extent of *Campylobacter* contamination of hatchery eggs, eggs from a commercial hatchery supplying chicks to broiler grow-out farms were tested. Hatchery eggs were supplied by multiple breeder flocks with different management conditions, and whether the breeder flocks were colonized by *Campylobacter* at the time of egg collection was not known. A total of 1,000 eggs at monthly intervals (100 eggs each time) were obtained from the hatchery and tested for *Campylobacter*. The eggs were <10 days old, and subjected to standard cleaning procedures (washing and disinfecting with a detergent) used routinely in commercial hatching facilities. To determine the contamination of eggs with *Campylobacter*, egg contents and shell membranes were aseptically separated, and the contents or the membranes from 10 individual eggs were pooled together as one sample. Each sample type (egg content or shell membranes) was treated as described above to isolate *Campylobacter* using the optimized enrichment and PCR detection methods.

**Antimicrobial susceptibility testing:** Fluoroquinolone (FQ) MICs of *Campylobacter* isolates recovered from feces of commercial broiler breeders were determined using enrofloxacin E-test strips (AB Biodisk, Sweden) following the manufacturer’s instructions. None of the flocks had received FQ antibiotics at any time
throughout their life. A total of 63 *Campylobacter* isolates obtained from 5 different flocks were tested for susceptibility to enrofloxacin. Briefly, 100 µl of each fresh *Campylobacter* culture in MH broth was spread onto MH agar plates. After the inoculated agar surface was completely dry, E-test strips of enrofloxacin, were applied onto individual plates. Plates were cultured at 42°C under microaerophilic conditions for 24 h. MICs were read at the point of intersection between the clear zone edge and the E-test strip. Enrofloxacin sensitive and resistant *C. jejuni* S3B isolates described in a previous study (28) were used as internal controls. *Campylobacter* isolates with an enrofloxacin MIC of $\geq 4 \mu g ml^{-1}$ were regarded as resistant.

### 4.4 RESULTS

**Sensitive recovery of *C. jejuni* from spiked egg samples:** Following artificial inoculation of egg contents and shell membranes, *C. jejuni* could be efficiently recovered with the optimized methods (Table 1). As few as a single *C. jejuni* CFU spiked into the suspensions of egg contents or shell membranes in the enrichment broth could be recovered from both sample types following the enrichment method. Assuming that each egg has approximately 50 ml of contents, and 1 g of shell membranes, the detection limit of the enrichment method would theoretically reach *ca*, 2.5 CFU from egg contents and 1 CFU from shell membranes of an egg. For isolation from egg membranes, density gradient centrifugation using Percoll® of enrichment cultures increased the sensitivity of the detection method as compared with the direct plating of enrichment cultures, especially when low numbers of bacteria were used for the inoculation. Also, the PCR assay targeting a region of 16S rRNA gene specific for *C. jejuni* and *Camp. coli* was
100% in accordance with our culture method used, indicating that methods were sensitive enough to detect low numbers of *Campylobacter* in eggs following an enrichment step.

**Egg-penetration by *C. jejuni***: A total of 50 eggs from a commercial hatchery for broilers were used to investigate the ability of *C. jejuni* strain S3B to invade the eggshell using a temperature differential method, which mimics the cooling of a freshly laid egg. The presence of *C. jejuni* in eggs placed in an incubator to hatch, and in the newly hatched chicks was monitored. Of the eggs collected on days 2, 6, 10, and 15 of incubation, none (5 eggs per day) had the organism when individual eggs were tested separately for the presence of *C. jejuni* by both the culture and the PCR method. On incubation day 21, 17 chicks hatched, and were housed in a disinfected room for 6 weeks. Cloacal swabs were taken on day 1 of age and weekly thereafter to isolate the organism. *Campylobacter* was not isolated from any of the cloacal swabs throughout the study period.

**Survival of *C. jejuni* in eggs**: Eggs were injected with a relatively low dose of *C. jejuni* strain S3B into the different compartments, and kept for 14 days at 18°C, which is the approximate temperature at which fertile eggs are stored before being set into incubators at commercial operations. When injected into the yolk (ca. 4.5 X 10^2 CFU for each egg), *C. jejuni* survived for at least 14 days, even though the numbers of eggs with viable *Campylobacter* gradually declined with increased storage time (Table 2). *Campylobacter* organisms were isolated only after enrichment but not following the direct culture, in agreement with the inability of the organism to multiple at temperatures...
below 31°C (21). There was no difference in the survivability of the organism between egg yolks of broiler chickens (with Campylobacter-specific maternal antibody) and SPF hens (without Campylobacter-specific maternal antibody), indicating that Campylobacter antibodies present at high levels in the egg yolk (36) did not adversely affect the viability of C. jejuni.

In contrast to the egg yolk, when C. jejuni was injected into the albumen (ca. 7 X 10^2 CFU for each egg) of eggs laid by SPF hens that were stored at 18°C, viability was less than 8 days (Table 2). On day 5 post inoculation, only 3 of 5 eggs was culture positive following the enrichment. None of the cultured eggs yielded C. jejuni on the subsequent sampling days. Similarly, C. jejuni was unable to survive for a long time when inoculated into the air sac (ca. 7 X 10^2 CFU for each egg) of SPF eggs that were kept at 18°C. On day 5 after inoculation, only 1 of 5 eggs was culture positive after enrichment, and no Campylobacter was isolated on the following sampling days. Campylobacter was not detected at any time via direct culture of egg samples when inoculated into the albumen or the air sac, indicating that the organism was unable to propagate inside eggs at 18°C.

Detection of Campylobacter in eggs from experimentally-infected SPF laying hens: Oral inoculation of SPF White Leghorn laying hens at week 22 of age with C. jejuni strain S3B resulted in colonization and shedding within a week as determined by culturing of the organism from cloacal swabs. Eggs laid by Campylobacter-colonized chickens were collected over a period of a year, during which the persistence of colonization was ensured by multiple inoculations of the birds via drinking water with the
same *C. jejuni* strain. The shedding level (log_{10} CFU g^{-1} of feces) ranged between approximately 3.5 and 6.5 in the majority of chickens throughout the study as determined by culturing of freshly-collected fecal dropping at weekly intervals.

Of 500 freshly-laid eggs that were examined on the day of lay and/or collection, *Campylobacter* were detected in 3 of 65 pooled (5-10 eggs for each pool) whole egg samples by both the enrichment culture and the PCR method. Samples positive by culture also gave positive results with the PCR, indicating a 100% correlation between the detection techniques. Also, none of the culture-negative egg samples were positive with the PCR. A 100% sequence identity was found in *cmp* gene (coding for the major outer membrane protein) sequences among *Campylobacter* isolates recovered from feces of the laying hens and the positive egg samples, confirming the layers as the source of *Campylobacter* isolated from the eggs. Since we only tested the pooled whole eggs, whether *Campylobacter* recovered from the eggs was on the surface or inside the egg was not known. In contrast to fresh eggs, *Campylobacter* was not detected in any of 800 eggs (80 pools; 10 eggs in each pool) that were first stored at 18°C for 7 days before testing by enrichment culture and PCR. In two instances when the level of *Campylobacter* shedding was high (ca. log_{10} 6.5 CFU g^{-1} of feces), a total of 140 eggs were placed in an incubator for hatching. *Campylobacter* was not isolated from any of the 95 newly hatched chicks that were tested on day 2 of age via cloacal swabbing.

**Prevalence of *Campylobacter* in breeder flocks and eggs:** All of the 5 broiler breeder flocks were tested positive for *Campylobacter* colonization. As shown in Table 3, prevalence of *Campylobacter* was generally high reaching 100% in some flocks, even
though the prevalence was relatively less in other flocks. In individual birds, the shedding level ranged between $\log_{10} 2.5$ and $6.5 \text{ CFU g}^{-1}$ of feces. There was no correlation between the age of the flock and the prevalence of *Campylobacter* colonization. Within 2 weeks of collection of fecal droppings from the breeder flocks, a total of 500 freshly laid (<2 days) floor and nest eggs (100 eggs from each flock) were tested for *Campylobacter* contamination. Fecal contamination was evident on the surface of most eggs, which did not have any cleaning treatment before examination. *Campylobacter* was not detected from any pooled whole egg samples (50 pools; 10 eggs for each pool) by either enrichment culture or PCR method.

**FQ MICs of *Campylobacter* isolates from breeder flocks:** A total of 63 *Campylobacter* isolates recovered from fecal droppings of different breeder flocks were all found susceptible to enrofloxacin as determined by Etest (results not shown). The MICs in all of the isolates were $<0.125 \text{ µg ml}^{-1}$. Laboratory studies demonstrated that use of FQ in chickens can rapidly select for FQ-resistant *Campylobacter* organisms (28). None of the breeder flocks surveyed in this study had used FQ antibiotics throughout the life of the flocks suggesting that emergence of FQ resistant *Campylobacter* may be directly related to use of FQ antibiotics on poultry farms.

**Detection of *Campylobacter* in eggs from a commercial hatchery:** The presence of *Campylobacter* was tested separately in the shell membranes and contents of a total of 1,000 eggs obtained from a commercial hatchery over a period of a year.
Campylobacter was not isolated from any of the pooled samples (10 eggs for each pool) by either the culture or the PCR method.

4.5 DISCUSSION

In the present study, we investigated the possibility of vertical (egg-borne) transmission in the introduction of Campylobacter into broiler flocks using both laboratory and field studies. The ability of Campylobacter to penetrate the egg shell, its viability inside the egg, and its prevalence in eggs from different sources were investigated using the optimized detection methods. Egg penetration studies indicated that Campylobacter was either unable to invade eggshell or could not remain viable beyond 2 days inside the incubating eggs, even if penetration had occurred. Using laying hens experimentally-infected with C. jejuni it was shown that Campylobacter contamination of eggs could occur at a low level, but survival of Campylobacter on or inside eggs was poor, suggesting that egg contamination by feces is not a pathway for Campylobacter transmission from hens to progenies. The lack of Campylobacter in eggs from commercial breeders that were colonized with Campylobacter and from a hatchery further disputes the role of egg-borne transmission in introducing Campylobacter into chicken flocks.

In general, vertical transmission of bacteria including Campylobacter may take place by primary (contamination of the egg content in the hen’s reproductive tract during the egg development) or secondary (contamination of the eggshell after the lay via fecal material containing the bacteria with the subsequent penetration of the agent inside the egg) infection of the egg. If vertical transmission of Campylobacter through the
secondary egg infection occurs, the organism must first penetrate through the eggshell and then maintain its viability once inside the egg until hatching. The limited ability of *C. jejuni* to penetrate the eggshell under laboratory conditions has been demonstrated in a number of earlier studies (3, 10, 17, 32, 37). Most of these investigators used a temperature differential method of various forms to test the penetration of *Campylobacter*. Although the temperature differential method theoretically mimics the events during cooling of freshly laid eggs, the conditions employed in these studies, such as use of broth or autoclaved feces contaminated with extremely high numbers of *Campylobacter*, during the assay may not necessarily simulate the environment of a newly laid egg. In reality, *Campylobacter* (and other bacteria) must compete effectively with other microflora present at high numbers in feces to penetrate through the eggshell. Therefore, we used freshly collected fecal material obtained from *Campylobacter*-free SPF hens without any further treatment (such as autoclaving) to closely mimic the process of natural egg contamination through the secondary egg infection. Following the temperature differential method, *Campylobacter* was not isolated from any of the incubating eggs that were first cultured on day 2 of incubation, indicating that either it was unable to penetrate the eggshell or it did not remain viable for 48 h in incubating eggs. Similarly, an earlier study by Neill *et al.* (32) showed that even though shell penetration by *C. jejuni* occurred following a temperature differential method, the organism was unable to survive for more than 6 h in any part of the eggs that were incubated in a humidified and ventilated atmosphere at 37 °C. However, in rare cases *Campylobacter* managed to survive inside the eggs following a temperature differential assay (10;11) or direct injection into the albumen (38) for long enough in incubating eggs.
to contaminate a low percentage of new hatchlings. Despite the observations (10, 11, 38), many other investigators indicated a short period of survivability (<72 h) of *Campylobacter* inside eggs (12, 17, 29, 32, 37). Shane *et al.* (37) showed that although contamination of commercial table eggs with feces containing *C. jejuni* resulted in a low level of penetration, viability of the organism on the shell surface was retained for only 16 h and the organism was not recovered from inside the eggs beyond 2 h when the eggs were kept at room temperature. Similarly, Doyle (17) using a temperature differential method demonstrated that even though *C. jejuni* could be isolated at a low level from the inner shell surface and shell membranes for up to 72 h, the organism was never recovered from the contents of eggs that were kept at 4°C. The high susceptibility of *Campylobacter* to the desiccation and atmospheric oxygen (33) are thought to be the primary reason for its low survivability on the egg surface. The absence of *Campylobacter* in the chicks hatched from the surface challenged eggs (via the temperature differential method) further supports the notion that *Campylobacter* contamination of eggs by a secondary egg infection is rare, if it occurs at all, and is unlikely to result in chicks infected with *Campylobacter*.

In commercial settings, newly laid eggs are usually stored up to 10 days at *ca.* 18°C before set into the incubators. The question, therefore, remains as to whether *Campylobacter*, when present in eggs, could stay viable during this storage period. To test this, different compartments (the yolk, the albumen, and the air sac) of the egg was artificially inoculated, and stored at 18°C for 14 days. Although the organism remained viable in egg yolk for up to 14 days, the survivability of *Campylobacter* inside the albumen and the air sac was less than 8 days. Previous studies also indicated the lower
survival rate of *C. jejuni* in the albumen as compared with the egg yolk at different
temperatures (12, 20, 29). It is likely that viability of *Campylobacter* was adversely
affected by the high pH and the presence of bactericidal compounds such as lysozyme
and conalbumen in the albumen. In general, bacterial penetration of the egg is most likely
to occur via the blunt (broad) end (where the air sac is located), since more pores in larger
diameter are found at this site of the eggshell, and the suction effect when the air sac is
formed could ease the bacterial penetration (30). Our results obtained from inoculation of
the air sac indicated that *Campylobacter* is unlikely to remain viable inside the egg at
18°C even if it penetrated the eggshell over the air sac. The low viability in the air sac is
probably due to the high sensitivity of the organism to atmospheric oxygen (33). The
inability of *Campylobacter* to penetrate the eggshell and/or to survive inside the egg for
longer than 48 h, and its low survivability inside the albumen and air sac of inoculated
eggs observed in this study would further reduce the likelihood of egg-borne transmission
of *Campylobacter*. However, the long survival rate of *Campylobacter* in the yolk may
have ramifications for vertical transmission. In general, primary contamination of the egg
yolk with bacteria occurs during egg development in the ovaries. *C. jejuni* has been
isolated from the reproductive tract of healthy laying and broiler breeder hens (6, 7, 24).
Therefore, egg yolk contamination by *C. jejuni* via an infection of the organs involved in
egg development is a possibility. However, this seems unlikely to happen under
commercial production conditions because many studies including the present one were
unable to detect live *Campylobacter* in the contents of hatching quality chicken eggs.

Apart from a very low level of isolation from the outer (17) or inner (38) surface
of freshly-laid (<14 h) non-hatching quality (soiled and/or cracked) eggs, so far live
Campylobacter has not been isolated from internal contents of sound (uncracked, hatching-quality) eggs laid by Campylobacter-shedding hens (1, 17, 24, 38). In the present study, we surveyed eggs obtained from different sources to better determine the association of Campylobacter with the eggs. Results obtained from SPF laying hens inoculated with C. jejuni showed that although a low percentage (4.6%) of newly-laid eggs could be contaminated with Campylobacter, storage of the eggs at 18°C for 7 days after laying resulted in eggs without Campylobacter-contamination at any site, suggesting that the few Campylobacter-positive eggs were likely the result of egg surface contamination. It is also possible that Campylobacter, if penetration had occurred, was unable to survive inside eggs during this storage period since viability of the organism following injection into the albumen or the air sac was less than 8 days (Table 2). Similarly, Clark and Bueschkens (11) showed that storage of experimentally infected eggs at 16°C for 5 to 8 days before being set into incubators led to absence of hatched chicks carrying C. jejuni, although 4% of chicks at hatching were infected when no storage was applied. In the present study we were also unable to isolate Campylobacter from commercial eggs (<2 days old) obtained from multiple, Campylobacter-colonized broiler breeder flocks even though fecal contamination of eggshell was apparent on most eggs, and the hens were still likely to be shedding Campylobacter at the time of egg collection. Although these eggs were not chilled, it is likely that any Campylobacter potentially present on the egg surface would not survive for a long time due to its sensitivity to desiccation and oxygen. In agreement with a previous study (38), we did not find Campylobacter in eggs obtained from a commercial hatchery for broilers. Routinely employed husbandry practices at hatcheries such as egg washing and/or fumigation, and
storage of eggs at cool temperatures before incubation are likely to be formidable barriers for *Campylobacter* survivability. Thus, vertical transmission of *Campylobacter* via eggs does not seem to play a role in introduction of *Campylobacter* into chicken flocks.

### 4.6 ACKNOWLEDGMENTS

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### 4.7 REFERENCES


The numbers represent the estimated CFUs that were spiked into 10 ml of egg content or 5 g eggshell membranes suspended in 90 ml or 45 ml enrichment broth, respectively.

**Table 4.1** Detection of *C. jejuni* in artificially-inoculated eggs using culture and PCR.

<table>
<thead>
<tr>
<th>spiking dose (CFU)*</th>
<th>egg contents</th>
<th>eggshell membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>culture</td>
<td>culture + Percoll</td>
</tr>
<tr>
<td>56</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*The numbers represent the estimated CFUs that were spiked into 10 ml of egg content or 5 g eggshell membranes suspended in 90 ml or 45 ml enrichment broth, respectively.*
### Presence of *C. jejuni* after inoculation on days

<table>
<thead>
<tr>
<th>Injection site</th>
<th>Inoculum dose (CFU/egg)</th>
<th>Presence of <em>C. jejuni</em> after inoculation on days:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg yolk</td>
<td>4.5x10^2</td>
<td>5/5* 5/5 5/5 4/5 3/5</td>
</tr>
<tr>
<td>Albumen</td>
<td>7.0x10^2</td>
<td>5/5 3/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>Air sac</td>
<td>7.0x10^2</td>
<td>4/5 1/5 0/5 0/5 0/5</td>
</tr>
</tbody>
</table>

\*Numbers of *Campylobacter*-positive eggs/total numbers of eggs examined.

**Table 4.2** Survivability of *C. jejuni* following injection into the egg yolk, albumen, and air sac of SPF eggs stored at 18°C.
<table>
<thead>
<tr>
<th>Flock no</th>
<th>Age* (week)</th>
<th>Prevalence in</th>
<th>Average Shedding level‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fecal droppings</td>
<td>Eggs</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>40/50†</td>
<td>0/100†</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>25/50</td>
<td>0/100</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>46/50</td>
<td>0/100</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>50/50</td>
<td>0/100</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>50/50</td>
<td>0/100</td>
</tr>
</tbody>
</table>

*Flock age at the time of collection of fecal samples.
†Number of *Campylobacter*-positive samples/total number of samples tested.
‡Mean log$_{10}$ CFU/g fecal droppings of *Campylobacter*-colonized birds.

**Table 4.3** Prevalence of *Campylobacter* in fecal droppings and eggs from 5 different commercial broiler breeder flocks
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