TRANSMISSION OF *CAMPYLOBACTER JEJUNI* IN BROILER CHICKENS

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ABSTRACT

Investigations were carried out aimed at identifying potential sources of transmission of *Campylobacter jejuni* to broiler chickens, determining whether boots can mechanically transmit *C. jejuni* to susceptible chickens, determining the transmission rate of *C. jejuni* in broiler chickens, and assessing the effect of washing slaughterhouse fomites with chlorinated water on the level of *C. jejuni* contamination during the day.

Bacteriological culture of samples taken in the premises of a broiler slaughterhouse showed that after washing and just before departure for depopulation of broiler farms, 75%, 58.33%, 47.22%, 50%, 54.29%, 66.67%, 31.25%, and 0% of pallets, crates, truck beds, truck wheels, drivers' boots, catchers' boots, forklift wheels, and tractor wheels respectively, were contaminated with *C. jejuni*. Therefore it was concluded that slaughterhouse personnel and fomites could potentially transmit *C. jejuni* during partial depopulation of broiler flocks.

The level of *C. jejuni* in consecutive sample batches of fomites (n = 30) that were collected in six periods of one hour each day (hence six batches), between 0530 and 1230 hrs, over six days, was found to range from 44.83% in the 1st batch and 66.67% in the 5th batch, indicating that washing fomites with pressurized chlorinated water makes no statistically significant difference ($p>0.05$) in the contamination level. The fomites sampled were pallets, crates, truck bed, truck wheels, and drivers' boots.

Susceptible broiler chickens became colonized with *C. jejuni* after exposure to boots that had been worn in a pen with infected chickens demonstrating that boots can mechanically cause horizontal transmission of the bacterium.

A transmission study conducted by introducing a bird infected with *C. jejuni* to 99 susceptible chickens demonstrated a rapid spread of the infection to all the birds within 48 hours and a transmission rate best represented by a simple epidemic curve. A transmission parameter beta (β), with a value of $2.1 \times 10^{-3} \pm 0.013 \times 10^{-3}$ (mean ±

standard error), was calculated by fitting the data into a simple deterministic epidemic model. The transmission rate in a population of 100 birds, predicted by this model, was very similar to that obtained in the experiment. Therefore, it was concluded that the transmission rate of *C. jejuni* in broilers resembles a simple epidemic.

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CHAPTER 1

1. INTRODUCTION

Epidemiological evidence has established that poultry meat, particularly meat from broiler chickens, is an important food source of human *Campylobacter jejuni* infections (Skirrow, 1991; Friedman *et al.*, 2000; Humphrey *et al.*, 2001). As a result, considerable research work has been carried out to identify both the sources and the routes of *C. jejuni* transmission to broiler chickens in order to develop preventive and control measures that would allow the production of *Campylobacter* free broilers. Despite such work, the sources of *C. jejuni* and the routes of introduction of the organism to broiler flocks are still uncertain (Gregory *et al.*, 1997; Shreeve *et al.*, 2000; Pattison, 2001).

The entry of catching crews into broiler houses to 'thin' flocks when the birds reach slaughter age, the so-called partial depopulation or 'first cut', has been associated with an increase in the risk of occurrence of *C. jejuni* in broiler flocks (Hald *et al.*, 2000; Wedderkopp *et al.*, 2000; Pattison, 2001). Moreover, investigators have demonstrated to a limited extend that the footwear of slaughterhouse personnel and fomites such as transport trucks, tractors, forklifts, crates, and pallets could be contaminated with *C. jejuni* (Berndtson *et al.*, 1996; Jacobs-Reitsma, 1997; Giessen *et al.*, 1998). Therefore an investigation was carried out at a slaughterhouse, using a bacteriological culture method to determine whether the slaughterhouse fomites stated above, are potential sources of *C. jejuni* for broiler chickens. In the same study, the slaughterhouse procedure for cleaning the fomites was assessed for its effect on the level of *C. jejuni* contamination of the fomites.

Considering that *C. jejuni* has reportedly been isolated from farmers' boots and epidemiologically associated with the introduction of the infection to broiler chickens (Lindblom *et al.*, 1986; Annan-Prah and Janc, 1988; Hald *et al.*, 2000), an experiment was carried out to determine whether boots could mechanically transmit *C. jejuni* from infected to non-infected broiler chickens. The design of the experiment

simulated on-farm practice where the farmer may wear boots in a pen with infected birds and shortly afterwards, enter another pen with susceptible birds using the same boots without disinfecting them.

Longitudinal epidemiological studies have demonstrated that *C. jejuni* spreads rapidly in broiler chickens to the extent that most birds in a flock become colonized within a week of the first evidence of infection (Smitherman *et al.*, 1984; Genigeorgis *et al.*, 1986; Shreeve *et al.*, 2000). However, these studies do not accurately determine the time of entry of *C. jejuni* into the broiler house and therefore do not provide an accurate quantitative measure of the transmission rate. An experimental transmission study was therefore carried out to determine the transmission rate of *C. jejuni* in a group of susceptible broiler chickens when an infected 'seeder' bird is introduced to them. The birds were reared to slaughter age under conditions similar to those in commercial farms, and the experiment was conducted at point of slaughter, which coincides with the time when *C. jejuni* is more likely to be detected for the first time in broiler flocks (Evans and Sayers, 2000).

CHAPTER 2

2. LITERATURE REVIEW

2.1 HISTORY AND TAXONOMY OF CAMPYLOBACTERS

The first major description of bacterial pathogens currently known as campylobacters was produced by MacFadyean and Stockman in 1913. The bacteria, then described as *Vibrio*, were isolated as early as 1906 and were experimentally shown to induce abortion in cattle and sheep. MacFadyean and Stockman (1913) considered their isolates of *Vibrio* to be the cause of epizootic abortion in cattle and sheep in Great Britain. Smith (1918) isolated microaerophilic *Vibrio* from foetal membranes of cows that had aborted in an outbreak of infectious abortion. Smith and Taylor (1919) reported that the microorganism was similar to that of Macfadyean and Stockman (1913), and named it *Vibrio fetus.*

Jones and Little (1931) isolated *Vibrio* organisms from faeces of calves with diarrhoea. They reported both acute and chronic enteritis in experimentally infected calves. Further studies of the isolates by Jones *et al.*, (1931) showed that they were serologically different from *Vibrio fetus.* The organism was recovered from intestinal mucosa. It had been observed that its predilection site, in cases of acute infection, was the jejunum. Therefore, Jones and his co-workers (1931) named it *Vibrio jejuni.*

A *Vibrio* sp., morphologically similar to *Vibrio fetus,* was isolated from the intestines of pigs with dysentery (Doyle, 1944). When fed to pigs, the *Vibrio* induced diarrhoea and could be recovered from the stools and portions of the colonic mucosa. Based on these findings, Doyle (1948) suggested the name *Vibrio coli* for the organism.

Cultures of *Vibrio* organisms were made from human patients (Levy, 1946; Ward, 1948; King, 1957). King (1957) compared human isolates, mostly from children with gastroenteritis, with *Vibrio fetus.* She found that 'related vibrios', as human isolates

were called, to grow at 37°C and 42°C and not at 25°C. On the other hand, *Vibrio fetus* grew at 25°C but not at 42°C. She also found 'related vibrios' serologically different from *Vibrio fetus*. King (1957) speculated that 'related vibrios' were similar to *Vibrio jejuni*.

There were reports of vibrios being found and isolated from poultry cases of infectious hepatitis (Hofstad *et al.*, 1958; Peckman, 1958; Truscott and Stockdale, 1965). An isolate from a liver of a chicken with avian vibrionic hepatitis was compared to 'related vibrios' of King (1957) and was found to be similar to these (Peckman, 1958).

Florent (1959) divided *Vibrio fetus* into *Vibrio fetus venerealis* and *Vibrio fetus intestinalis*. This classification was based on the finding that *Vibrio fetus venerealis* was transmitted through mating and was therefore associated with infertility whereas *Vibrio fetus intestinalis* was not sexually transmitted and did not cause infertility. It was found in the intestines, and was thought to be transmitted by the faecal oral route.

Temperature tolerance tests of 15 *Vibrio fetus intestinalis* isolates of ovine abortion, classified as serotype I, showed that they grew at 37°C and 42°C but not at 25°C (Firehammer and Berg, 1965). A human 'related vibrio' studied by the same workers was found to be similar to *Vibrio fetus intestinalis*. They speculated that some of the 'related vibrios' from humans could originally have come from sheep.

A new genus called *Campylobacter* (Greek for curved rod) was created by Sebald and Veron in 1963. Sebald and Veron (1963) did deoxyribonucleic acid (DNA) base composition analyses of *Vibrio* species. They found that the guanine plus cytosine (G+C) content of the DNA of *Vibrio fetus* ranged between 29 and 36 mole % whereas that of *Vibrio cholerae* ranged between 40 and 53 mole %. It was also known that *Vibrio fetus* did not ferment sugars while *Vibrio cholerae*, the type species of the genus *Vibrio*, was fermentative. Davis and Park (1962) had already expressed the need for reclassification of species of the genus *Vibrio* on the grounds that the species were so varied in characteristics that it was impossible to exclusively define the genus. Sebald and Veron (1963) moved to the genus *Campylobacter*, *Vibrio fetus*, *Vibrio*

bubulus, *Vibrio jejuni* and *Vibrio coli*. They suggested *Campylobacter fetus* as the type species of the genus.

While studying the cultural characteristics of 1220 *Campylobacter* strains, Skirrow and Benjamin (1980b) found a group of strains (42) that were thermophilic but unlike *C. jejuni* and *C. coli,* were resistant to nalidixic acid. The isolates were mostly from wild sea gulls and had several cultural characteristics in common. They were designated nalidixic acid resistant thermophilic campylobacters (NARTC) as they were different from known species (Skirrow and Benjamin 1980a; 1980b). Ten strains of NARTC showed a high DNA base sequence relatedness (≥ 76%) within the group while showing a low base sequence relatedness (≤ 15%) to other *Campylobacter* species including *C. jejuni* and *C. coli* (Benjamin *et al.*, 1983). As a result Benjamin *et al.*, (1983) proposed the name *Campylobacter laridis* for NARTC. The name of the species has since changed to *Campylobacter lari* (von Graeventz, 1990).

According to Loesche *et al.*, (1965) a *Vibrio* was isolated from the sputum of a human patient with acute bronchitis by Tunnicliff in 1914 and it was named *Vibrio sputorum* by Prevot in 1940. Florent (1953, cited by Loesche *et al.*, 1965) named a non - pathogenic *Vibrio* isolate from the genital tract of cattle *Vibrio bubulus*. *Vibrio sputorum* and *Vibrio bubulus* were found to have similar phenotypic and biochemical characteristics and were reclassified as one species *Campylobacter sputorum*, with two subspecies namely *Campylobacter sputorum* subsp. *sputorum* (Prevot 1940) and *Campylobacter sputorum* subsp. *bubulus* (Florent 1953) (Veron and Chatelain, 1973). The species *Campylobacter sputorum* was different from other species in that it was catalase-negative and was considered non-pathogenic for humans and animals (Penner, 1988).

A non-pathogenic catalase-positive *Vibrio* was isolated from the faeces of sheep and named *Vibrio fecalis* by Firehammer in 1965. Studies later showed that the organism had many phenotypic characteristics in common with catalase-negative campylobacters (Harvey and Greenwood, 1983; Roop *et al.*, 1984; Roop *et al.*, 1985). DNA homology studies by Roop *et al.*, (1985) showed that "*Campylobacter fecalis*",

as it was later called, was a subspecies of *Campylobacter sputorum*. They proposed the name *Campylobacter sputorum* subsp. *fecalis* for it.

Rowland and Lawson (1974) microscopically observed bacteria in the apical cytoplasm of glandular intestinal epithelium of pigs suffering from porcine intestinal adenomatosis (PIA). Subsequently they isolated campylobacters from lesions of PIA and proposed the name *Campylobacter sputorum* subsp. *mucosalis* for the organisms (Lawson and Rowland, 1974; Lawson *et al.*, 1975). A proposal was made for the name to be revived by Lawson *et al.*, (1981) after it was excluded from the approved lists of bacterial names (A.L.B.N) (Skerman *et al.*, 1980). However, Roop *et al.*, (1985) found the organisms to be different from *C. sputorum* by DNA homology studies and they proposed the name *Campylobacter mucosalis* for them.

Tanner *et al.*, (1981) did a taxonomic study of 46 Gram negative, asaccharolytic, rod shaped bacteria from lesions of periodontal disease in humans. They found six *Campylobacter* strains that could be differentiated from previously described *Campylobacter* spp and reference strains of other genera normally found in the oral cavity. The isolates had a G + C content of DNA of 34 to 38 mole %, showed a 100% DNA homology with each other, and could be differentiated by phenotypic tests from other *Campylobacter* spp. Tanner *et al.*, (1981) proposed the name *Campylobacter concisus* for the organisms. They also proposed that the G + C content of DNA of the genus *Campylobacter* be raised from a maximum of 36 mole % to 38 mole % to include all *C. concisus* isolates. Roop *et al.*, (1985) confirmed by DNA hybridization studies that *C. concisus* isolates were a separate homologous group. Tanner (1986) used protein profiles to differentiate *C. concisus* from *Wolinella* spp.

Another outcome of the taxonomic study by Tanner *et al.*, (1981) was the creation of the genus *Wolinella*, which included strains with a G + C content of DNA of 42 to 49 mole %. Two groups of bacteria with similar phenotypic properties but forming distinct groups in DNA–DNA hybridization studies, were named *Wolinella*

succinogenes (the type species) and *Wolinella recta*. A new species, *Bacteroides gracilis*, was proposed for another group of non-motile, oxidase-negative bacteria with a G + C content of DNA ranging from 44 to 46 mol % (Tanner *et al.*, 1981).

New strains of Gram negative vibrio-shaped bacteria, also isolated from the human oral cavity, were found to be different from known species by DNA-DNA homology experiments (Tanner *et al.*, 1984). They were assigned to a new species *Wolinella curva* (Tanner *et al.*, 1984). At this point, it was widely acknowledged that the phenotypic properties of the bacteria in the genus *Wolinella* were similar to those of *Campylobacter*. The two genera differed in that the G + C content of DNA of *Wolinella* was higher than that of *Campylobacter* (Tanner *et al.*, 1984).

Further studies to characterize bacteria of the genera commonly found in the human oral cavity, using protein profiles of sonicated whole cells, confirmed that *W. recta*, *W. curva*, and *B. gracilis* were distinct species (Tanner, 1986). However, Paster and Dewhirst (1988) found that the three species clustered with true campylobacters with an interspecies homology of 94.4% or greater. They had compared the 16S ribosomal ribonucleic acid (rRNA) sequences of each of the species with those of *Campylobacter* and related genera. When the genus *Campylobacter* was emended so that the G + C content of DNA of organisms in it had a range of 30 to 46 mol %, *W. recta* and *W. curva* were reclassified as *Campylobacter* rectus and *Campylobacter curvus* respectively (Vandamme *et al.*, 1991). Meanwhile Han *et al.*, (1991) reported that *W. recta*, *W.curva*, and *B. gracilis* were microaerophilic rather than anaerobic as it was previously believed.

Vandamme *et al.*, (1995) found that the fatty acid composition of *B. gracilis* was similar to that of campylobacters. They transferred the bacterium to the genus *Campylobacter* to become *Campylobacter gracilis*.

Etoh *et al.*, (1993) proposed a new name *Campylobacter showae* for nine campylobacter-like strains isolated from the human gingival crevices. They had found the strains to be a distinct group when compared to species of the genus *Campylobacter*, *Wolinella*, *Bacteroides* and *Helicobacter,* using 16S rRNA sequence similarity data. The strains could also be distinguished from known species by their unique whole-cell protein profiles, western blot pattern and the high intra-group DNA homology, which was greater than 74 %. As the strains possessed two to five flagellae, Etoh *et al.*, (1993) suggested that the genus *Campylobacter* be emended to include bacteria with multiple flagella.

Catalase-positive strains of *Campylobacter* organisms were isolated together with the catalase-negative *C. sputorum* subsp. *mucosalis* from lesions of swine proliferative ileitis (Gebhart *et al.*, 1983). The organisms could be differentiated from other species of *Campylobacter* by biochemical tests. Based on phenotypic characteristics, the organisms were thought to be closely related to *C. fetus* subsp. *intestinalis.* Therefore Gebhart *et al.*, (1983) proposed that they should be called *Campylobacter hyointestinalis.*

On *et al.*, (1995) carried out a detailed study of *Campylobacter hyointestinalis* strains using both phenotypic and genomic methods. They found that some strains were genomically homogeneous but distinct from reference strains of *C. hyointestinalis* and they referred to them as *Campylobacter hyointestinalis*-like or "CHY" group. The level of DNA relatedness within the "CHY" group was 84 to 90 %. However, they were still significantly related to reference strains of *C. hyointestinalis* at levels of 56 to 71 %. A new subspecies *Campylobacter hyointestinalis* subsp. *lawsonii* was proposed for the "CHY" group of strains. The other strains were to be known as *Campylobacter hyointestinalis* subsp. *hyointestinalis* (On *et al.*, 1995).

A study of *Campylobacter* species in dogs resulted in the isolation of 63 thermophilic strains from both normal and diarrhoeic dogs (Sandstedt *et al.*, 1983). The isolates showed catalase activity after 30 seconds rather than 10 seconds. Therefore, they were

described as catalase-negative or weak (CNW) by the same workers. Otherwise, their phenotypic characteristics were not distinct from those of known *Campylobacter* species. However, they showed a high (> 80%) DNA homology with each other while being related to *C. jejuni* and *C. coli* at about 40 percent (Sandstedt *et al.*, 1983). Following widespread isolation of CNW strains from humans, dogs and cats, Sandstedt and Ursing (1991) proposed the name *Campylobacter upsaliensis* for the organisms although there was still a lack of distinct phenotypic characteristics to differentiate them from known thermophilic campylobasters.

Campylobacter helveticus was proposed as a new species for a group of catalase-negative thermophilic bacterial strains isolated from both diarrhoeic and normal dogs and cats (Stanley *et al.*, 1992). The strains had been found to be phenotypically indistinguishable from *C. upsaliensis* but they did not react with *C. upsaliensis* in DNA–DNA hybridization tests. In addition, the group had a homogeneous but unique whole-cell protein profiles, and ribosomal RNA (both 16S and 23S) gene profiles, and its DNA homologies were distinct from those of previously described *Campylobacter* species (Stanley *et al.*, 1992). *C. helveticus* was reported by the same workers to be closely related to *C. upsaliensis* .

Recently, a new species named *Campylobacter lanienae* was described by Logan and others (2000). The description was based on two bacterial strains isolated from the faeces of healthy abattoir workers and identified by polymerase chain reaction (PCR) as campylobacters. Further studies of the strains using nucleotide sequence analysis of 16S rRNA gene, and DNA–DNA homology tests indicated that they had no significant homology with any other species of the genus *Campylobacter, Helicobacter* and *Arcobacter* (Logan *et al.*, 2000)

Table 2.1 The taxonomic status of the genus *Campylobacter*

Present classification	Previous classification	Remarks
C. fetus subsp.*fetus*	*C. fetus* subsp. *intestinalis*	-
C.fetus subsp. *venerealis*	-	-
C. jejuni subsp. *jejuni*	*Vibrio jejuni*	-
C. jejuni subsp. *doylei*	-	Nitrate- negative strains of *C. jejuni* (Steele and Owen, 1988)
C. coli	*Vibrio coli*	Include *C. hyoilei* (Alderton *et al.*, 1995)
C. sputorum bv. *sputorum*	-	Include *C. sputorum* bv. *bubulus* (On *et al.*, 1998a)
C. sputorum bv. *fecalis*	*Vibrio fecalis*	Catalase-positive strains of *C. sputorum* (On *et al.*, 1998a)
C. sputorum bv.*paraureolyticus*	-	Urease-positive strains of *C. sputorum* (On *et al.*, 1998a)
C. hyointestinalis subsp. *hyointestinalis*	-	-
C. hyointestinalis subsp. *lawsonii*	-	-
C. concisus	-	-
C. mucosalis	*C. sputorum* subsp. *mucosalis*	-
C. gracilis	*Bacteroides gracilis*	-
C. lari	*C. laridis*	Nalidixic acid resistant thermophilic campylobacters (NARTC)
C. upsaliensis	-	-
C. curvus	*Wolinella curva*	-
C. rectus	*Wolinella rectus*	-
C. showae	-	-
C. helveticus	-	-
C. lanienae	-	-

2.2 GENERAL CHARACTERISTICS OF *CAMPYLOBACTER JEJUNI*

2.2.1 The bacterium

Organisms of the species *Campylobacter jejuni* are slender, spirally curved, Gram negative rods that are 0.2 to 0.5 µm wide and 0.5 to 8 µm long (Smibert, 1984; Vandamme *et al.,* 1991). When two cells form short chains, they may appear gull-wing shaped. Cells in old cultures form spherical or coccoid bodies. When observed under a dark-field microscope, the cells are motile with a characteristic cork-screw-like darting motion. Cells have a single polar unsheathed flagellum at one or both ends. They are non-spore forming.

C. jejuni is oxidase producing and catalase-positive. However, some strains show a weak or negative catalase reaction (Steele and Owen, 1988). *C. jejuni* does not ferment or oxidise carbohydrates (Penner, 1988). It has a respiratory type of metabolism and uses amino acids and intermediates of the tri-carboxylic acid cycle. Strains of *C. jejuni* subspecies *jejuni* reduce nitrates while those of *C. jejuni* subspecies *doylei* do not reduce nitrates (Penner, 1988; Steele and Owen, 1988). *C jejuni* is hippurate positive by the tube test method of Harvey (1980). According to Penner (1988) 0.2% of the hippurate positive isolates did not have the properties of *C. jejuni,* which suggests that they could be falsely identified as *C. jejuni.* Hebert *et al.*, (1984) confirmed by DNA hybridization tests that a few strains of *C. jejuni* were hippurate negative.

The G + C content of DNA for *C. jejuni* ranges from 28 to 32 mole % (Smibert, 1984; Penner, 1988; Steele and Owen, 1988). This is at the lower range for the genus *Campylobacter*, which has a G + C content of DNA range of 28 to 46 mole % (Vandamme *et al.,* 1991). *C. jejuni* has menaquinone-6 and methyl-substituted menaquinone as the major respiratory quinones (Ursing *et al.*, 1994; Vandamme *et al.*, 1995). The major fatty acids found in *C. jejuni*, like in the genus *Campylobacter,* are tetradecanoic acid, hexadecanoic acid, hexadecenoic acid and octadecenoic acid (Blaser *et al.*, 1980a; Moss *et al.*, 1984; Vandamme *et al.,* 1991)

2.2.2 Pathogenesis and virulence

After entering the human body through ingestion, *C. jejuni* colonises the intestinal tract and causes gastroenteritis and in some cases systemic disease. The virulence factors that allow *C. jejuni* to cause an infection and the progression of the disease (pathogenesis) have been reviewed extensively (Walker *et al.,* 1986; Wallis, 1994; Ketley, 1997; Wassenaar, 1997). The virulence determinants are considered to be multi-factorial in nature with some still not well characterised. Those that have been identified and studied significantly include motility and chemo-taxis, adhesion to epithelial cells, cell invasion, and toxin production (Walker *et al.,* 1986; Wallis, 1994; Ketley, 1997; Wassenaar, 1997)

(i) Motility and chemo-taxis

Campylobacters are thought to penetrate the mucoid layer on the epithelium to colonise the mucosa assisted by the flagella and the spiral shape of the organisms (Lee *et al.,* 1986). The presence of the flagella in *Vibrio cholerae* for instance, has been reported to increase virulence by up to a 100-fold compared to non-motile mutants of the organisms (Yancey *et al.*, 1978). *C. jejuni* is chemo-tactic to mucin, a carbohydrate L-fucose, several amino acids, and some intermediates of the tri-carboxylic acid cycle (Walker *et al.*, 1986). The bacterium therefore, tends to go up the concentration gradient of these chemo-attractants, which is toward the mucus. Non-chemo-tactic mutants of *C. jejuni* failed to colonise suckling mouse intestines, demonstrating the importance of chemo-taxis in infection (Takara *et al.*, 1992).

(ii) Adhesion and invasion

It is assumed that *C. jejuni* adheres to intestinal cells before invading them. However, *in vitro* studies show that the organism adheres to tissue culture cells without subsequent invasion (Everest *et al.*, 1992). Studies also show that adhesion factors occur on the flagellum and the cell membrane (McBride and Newell, 1983). The outer membrane proteins (OMP) and lipopolysaccharides (LPS) have been described as adhesion factors that bind to eukaryotic cells (De Melo and Pechere, 1990). Doig *et al.*, (1996) observed fimbrae on *C. jejuni* with an electron microscope and speculated

that fimbrae constituted a virulence factor. *C. jejuni* is able to invade tissue culture cells (Konkel and Joens, 1989; Everest *et al.*, 1992). Evidence of invasion is also demonstrated by inflammatory cell accumulation in the lamina propria and the presence of crypt abscesses in histological sections from human patients (Lee *et al.*, 1983). The presence of other pathogens such as entero-viruses is reported to enhance the invasiveness of *C. jejuni* (Bukholm and Kappend, 1987).

C. jejuni is also able to get from the mucosa to the mesenteric lymph nodes (a process called translocation) and to cause a bacteraemia (Wallis, 1994). The organism is speculated to get to the lymph nodes either through epithelial cells (trans-cytoses) or by passing between cells (para-cellular route) (Everest *et al.*, 1992; Ketley, 1997). Monocytes may also assist translocation since *C. jejuni* is known to survive within monocytes for six to seven days (Walker *et al.*, 1986; Konkel and Joens, 1989).

(iii) Toxins

Campylobacter jejuni was reported by Johnson and Lior (1984) to produce two exotoxins: a heat–labile enterotoxin and a cytotoxin. The *C. jejuni* enterotoxin has some similarities to the *Vibrio cholera* toxin and the *Escherichia coli* heat-labile toxin (Ruiz-Palacious *et al.*, 1983; Saha *et al.*, 1988). The toxin binds to specific receptors (GM1 gangliosides) on the plasma membrane initiating a process that results in an increase in intracellular concentration of cyclic adenosine mono-phosphate and fluid secretion. The clinical manifestation of fluid secretion is watery diarrhoea. The cytotoxin produced by *C. jejuni* is thought to cause cell death by blocking protein synthesis, as does the *shiga*-toxin. It causes Chinese hamster ovary cells to round up and die in *in vitro* experimental studies (Ruiz-Palacious *et al.*, 1983). Another toxin termed cyto-lethal distending toxin, was discovered by Johnson and Lior in 1988. It causes progressive cell distension followed by cell death in experiments using tissue culture cells. The role of the toxin in pathogenesis is still unknown.

2.2.3 Growth requirements.

Campylobacter jejuni is a fastidious organism that requires a complex medium for growth. It is asaccharolytic and utilises amino acids and intermediates of the citric acid cycle as energy sources (Hoffman *et al.,* 1979a; Leach *et al.*, 1997). *C. jejuni* is obligately microaerophilic with an aerobic respiratory metabolism. However its genome has been found to have genes for fumarate reductase, which suggests the possibility of anaerobic growth with fumarate as the terminal electron acceptor (Parkill *et al.*, 2000). Kelly (2001) speculated that the growth of *C. jejuni in vivo* could be anaerobic as the mammalian or avian gut it colonises is mainly anaerobic.

For growth, *C. jejuni* requires reduced oxygen levels (5–10% v/v), increased carbon dioxide levels (5-10% v/v), and nitrogen. Being thermophilic, the optimum temperature for culturing *C. jejuni* is 42°C. It does not grow at 25°C and above 45°C (Smibert, 1984). The organism is sensitive to an acidic environment and therefore does not grow at a low pH (pH< 3) (Blaser *et al.*, 1980b). Growth supplements are required for culturing *C. jejuni*. Blood or its derivatives such as serum albumen are often included in *Campylobacter* media (Leach *et al.,* 1997). Blood-free media containing charcoal as a growth supplement are able to sustain the growth of *C. jejuni* (Bolton *et al.,* 1984b). A supplement containing ferrous sulphate, sodium bisulfite, and sodium pyruvate (FBP supplement) was found to improve growth and aero-tolerance of *Campylobacter fetus* (George *et al.,* 1978). FBP supplement, blood, charcoal and other growth supplements that enhance growth and oxygen tolerance of *C. jejuni*, are thought to do so by removing super-oxide anions and hydrogen peroxide from the culture medium (Hoffman *et al.,* 1979b; Bolton *et al.,* 1984b; Kelly, 2001).

2.2.4 Isolation of *Campylobacter jejuni*

The samples commonly taken for the isolation of *C. jejuni* vary depending on the purpose for sampling. In poultry, faecal material or cloacal swabs are often taken to investigate flock prevalence. Other samples reportedly taken for epidemiological studies include feed, litter, water, air, meat, and swabs from the proventriculus, caecum, small intestines and large intestines (Pearson *et al.*, 1993; Wallace *et al.*, 1997; Stanley *et al.*, 1998). The use of transport media such as Cary- Blair medium is

recommended if a delay exceeding two hours before processing is envisaged (Misawa *et al.*, 2000). Drying, high temperatures, low pH, and exposure to atmospheric levels of oxygen can reduce recovery of campylobacters from samples (Goossens and Butzler, 1992).

There has been significant improvements in the methods used to isolate campylobacters since Dekeyser and others (1972) reported the first positive stool cultures. Their method involved filtering faeces through a 0.65 µm pore-sized filter on to blood agar. Skirrow (1977) replaced the filtration method with a blood-based solid-selective medium containing vancomycin, polymyxin and trimethoprim. Other similar media were subsequently developed and used to isolate *C. jejuni* and *C. coli* from diarrhoeic human patients (Bolton and Robertson, 1982; Goossens *et al.*, 1983; Goossens *et al.,* 1986). These media varied in their antibiotic supplements and they were often found to inhibit contaminating bacteria more than Skirrow's medium (Goossens and Butzler, 1992). Bolton *et al.*, (1983) carried out a comparative study of five solid antibiotic selective media for the isolation of *C. jejuni* and *C. coli* from faecal samples of humans, pigs, cattle, chicken, and drain effluents. The media were Skirrow's medium, Preston medium, Blaser's medium, Campy-BAP medium, and Mueller-Hinton agar. They found Preston medium to be the most selective.

Bolton *et al.*, (1984a) developed the first blood-free medium for the isolation of *Campylobacter jejuni*. In this medium, blood was replaced by charcoal, ferrous sulphate and sodium pyruvate. It was called charcoal - cefazolin-sodium deoxycholate agar (CCDA). Other similar media have since been developed such as charcoal based medium (CSM) and Modified Campylobacter Charcoal Deoxycholate agar (MCCDA) (Karmali *et al.*, 1986; Stanley *et al.*, 1998). The advantage of blood-free medium is that it is cheaper and quality control is easier compared to blood, which is likely to be variable and contaminated (Bolton *et al.*, 1984a).

Enrichment media are used to increase isolation rates of campylobacters in general by up to 20%. In one study, isolation rates were improved by 48% using Preston enrichment broth compared to direct plating (Bolton *et al.*, 1983). Enrichment is indicated when there is delayed transport of the samples to the laboratory, when the organisms are suspected to be few in the sample or sub-lethally injured (Humphrey *et*

al., 1989; Murray *et al.*, 1995). Enrichment media that have been in use include Preston enrichment broth, campy-thio, campylobacter enrichment broth, alkaline peptone water and Bruce-Zochowsky medium (Agulla *et al.,* 1987; Carter and Cole, 1990, Murray *et al.*, 1995). Different workers consistently recommend the inclusion of enrichment media in epidemiological studies of *C. jejuni* and *C. coli* (Bolton *et al.*, 1983; Garcia *et al.*, 1985; Stanley *et al.*, 1998). The enrichment medium is inoculated and incubated at 37°C for 4 hrs or in microaerophilic conditions at 42°C for 24 to 48 hrs before further culture (Stanley *et al.*, 1998).

Campylobacters found in poultry are thermophilic. For their isolation, inoculated selective media are incubated at 42°C for up to 72 hrs. Microaerophilic conditions of 5% O_2, 10% CO_2, and 85% N_2 are required and can be attained by infusion of gas mixtures from gas cylinders into an incubator or a gas generator envelope placed in an anaerobic jar without a catalyst. The plates are examined after 24 hrs and 48 hrs. The decision of growth or no growth is made at 72 hrs (Anonymous, 1984; Murray *et al.*, 1995). Suspect colonies can be sub-cultured onto blood agar to get a pure culture, which is used for identification or frozen at -70°C for future studies.

2.2.5 Identification of *C. jejuni*

Campylobacter species commonly found in poultry are *C. jejuni*, *C. coli* and *C. lari*. Campylobacters are differentiated from other bacteria by their characteristic colonial morphology. They form flat non-haemolytic, watery, grey colonies, which sometimes have a pink tinge. The colonies spread along streak marks. They can be round, convex, glistening and less spreading if there is little moisture in the medium (Carter and Cole, 1990). *Campylobacter coli* morphology was found to be occasionally atypical on an isolation medium called Campy-BAP making it difficult to differentiate the organism from coliforms (Bolton *et al.*, 1983). For microscopic examination of Gram stains, the use of carbol fuscin as the counter stain instead of safranin has been reported to make it easier to see the organisms (Stanley *et al.*, 1998). Motility should be observed using a phase contrast microscope (Garcia *et al.,* 1985).

There are few phenotypic characteristics that differentiate the three *Campylobacter* species found in poultry. All the three species are oxidase and catalase–positive.

Campylobacter jejuni hydrolyses sodium hippurate while *C. coli* does not and this property is used to differentiate the two species. *C. lari* is resistant to nalidixic acid which differentiates it from *C. jejuni* and *C. coli* (Skirrow and Benjamin, 1980b). However, a few strains of both *C. jejuni* and *C. coli* have been reported to be nalidixic acid resistant (Ursing *et al.*, 1983; Walder *et al.*, 1983; Lior, 1984). In the literature, there are a number of schemes, which can help characterise and differentiate campylobacters based on biochemical reactions and other phenotypic properties (Skirrow and Benjamin, 1980a; 1980b; Penner, 1988; Goossens and Butzler, 1992; Vandamme and Deley, 1991; Holt *et al.,* 1994). Table 2.2 shows characteristics that can be used to differentiate thermophilic campylobacters of avian origin. *C. fetus* subsp. *fetus* is included for comparison as it is the type species of the genus *Campylobacter.*

Table 2.2 A scheme for the differentiation of *C. jejuni*, *C. coli*, and *C. lari*.[a]

Species	Biochemical and other phenotypic properties											
	Test						Growth at or in:				Susceptibility to:	
	Catalase	oxidase	Nitrate	H_2S (TSI)[b]	Hippurate hydrolysis	Indoxyl-acetate hydrolysis	25° C	37°C	42°C	0.1% TMAO[c] (anaerobic)	Nalidixic acid	Cephalo-thin
C. fetus subsp. *fetus*	+[d]	+	+	-	-	-	+	+	-	-	R	S
C. jejuni subsp *jejuni*	+	+	+	-	+	+	-	+	+	-	S	R
C. jeuni subsp. *doylei*	v	+	-	-	v	+	-	+	w	-	S	S
C. coli	+	+	+	+	-	+	-	+	+	-	S	R
C. lari	+	+	-	+	-	-	-	+	+	+	R	R

[a] Data obtained from Benjamin *et al.*, (1983), Penner (1988), Steele and Owen (1988), Vandamme and Deley (1991), and Holt *et al.*, (1994).

[b] TSI, triple sugar iron agar.

[c] TMAO, trimethylamine-N-oxide hydrochloride.

[d] +, positive reaction; -,negative reaction; w, weak growth; v, variable reaction; S, susceptible; R, resistant.

2.2.6 Biotyping and serotyping of *C. jejuni*

The subtyping of *C. jejuni* by biotyping, serotyping, or a combination of both techniques is necessary for epidemiological studies of strains particularly those that cause human disease (Penner and Hennessy, 1980; Lior, 1984; Moran and Penner, 1999). Skirrow and Benjamin (1980a) reported two *C. jejuni* biotypes that they could differentiate using the hippurate hydrolysis test and a rapid test for H_2S production in iron media. Subsequently Lior (1984) reported a biotyping scheme that divided *C. jejuni* into four biotypes designated I, II, III, and IV. Bolton *et al.*, (1984c) developed a biotyping scheme that included tests in the Lior scheme. This scheme, known as the Preston biotyping scheme, could potentially identify 500 biotypes of *C. jejuni* (Klena, 2001). The disadvantage of biotyping is that inconsistent results may be obtained depending on the nature of the media used (On and Holmes, 1991).

There are two widely accepted serotyping schemes for *C. jejuni*. The method described by Penner and Henessy (1980) based on soluble heat–stable antigens initially identified 23 *C. jejuni* serotypes. It was later extended to include 42 serotypes (Moran and Penner, 1999). The other method by Lior *et al.*, (1982) based on heat–labile antigens could initially identify 21 *C. jejuni* serotypes. Later, it could identify 63 serotypes (Patton and Wachsmuth, 1992). The limitation of serotyping is that it is only available mainly in reference laboratories. Also, it is reported that between 4 and 20% of *C. jejuni* isolates are non-typeable even with the more widely used Penner serotyping scheme (Klena, 2001). Lior (1984) reported that combining serotyping with biotyping provided additional epidemiological information in the study of *C. jejuni* outbreaks.

2.2.7 Molecular typing of *C. jejuni*

Molecular typing schemes examine the relatively more stable chromosomal differences between bacterial strains and therefore they are considered more sensitive than phenotyping schemes (On *et al.*, 1998b). Klena (2001) reviewed molecular genetic techniques for typing *C. jejuni* and identified the following main categories; DNA hybridization methods, bacterial restriction enzyme DNA analysis (BRENDA), polymerase chain reaction (PCR) methods, random amplified polymorphic DNA

(RAPD) analysis, and multi-locus sequence typing (MLST). Pulsed-field gel electrophoresis (PFGE), which is one of several available genomic typing techniques was reported by Owen *et al.*, (1995) to be the most accepted for typing *Campylobacter* species. In this method, macro-restriction enzymes, commonly *Sma*I, *Kpn*I, and *Sal*I, are used to infrequently cut bacterial genomic DNA resulting in a few (≤ 10) large-molecular-weight chromosomal DNA fragments (Yan *et al.*, 1991; Tenover *et al.*, 1995; On *et al.*, 1998b). Subjecting the fragments to an electric field generates banding patterns (PFGE profiles) that can serve as a genetic 'finger-print' of the bacterial strain (Yan *et al.,* 1991).

The attributes of PFGE are that unlike biotyping and serotyping, it is highly reproducible and discriminatory (Tenover *et al.*, 1995; Gibson *et al.*, 1997; Harrington *et al.*, 1999; Klena, 2001). However, the technique requires specialized equipment, it is time–consuming and laborious and therefore unsuitable for typing a large number of isolates (De Boer *et al.*, 2000). PFGE has been used to demonstrate that each Penner serotype consists of a heterogeneous population of strains or pulsotypes (Harrington *et al.*, 1999). Identical *Sma*I fragment patterns have also been found in strains from two different Penner serotypes (HS50 and HS65)(Gibson *et al.*, (1997). The use of multiple genotyping techniques has been suggested as an option for achieving better strain differentiation (De Boer *et al.*, 2000). A number of workers recommend a combination of Penner serotyping for heat–stable antigens and PFGE for effective epidemiological typing of *C. jejuni* (Owen *et al.*, 1995*;* Harrington *et al.,* 1999).

2.3 EPIDEMIOLOGY

The understanding of the epidemiology of *C. jejuni* in broilers is considered necessary for reducing human campylobacteriosis associated with the consumption of poultry meat (Blaser *et al.,* 1983; Evans, 1992). Most of the investigations into the epidemiology of campylobacters in broilers have been aimed at identifying both animal and environmental reservoirs of the bacterium (Kapperud *et al.*, 1993). The studies have shown that *Campylobacter* species are widely spread and have numerous potential sources within and external to the poultry house environment (Deming *et al.*,

1987; Stern, 1992). Despite the identification of reservoirs of *C. jejuni,* the definitive sources of infection for broilers are yet to be established (Jones *et al.*, 1991; Nesbit *et al.*, 2001).

2.3.1 Animal reservoirs of *C. jejuni*

Many animals are carriers of *C. jejuni.* Reviews of documented isolation of *C. jejuni* from animals indicate that in food animals, the organism is found in cattle, pigs, sheep, and poultry (Blaser *et al.*, 1980b; Kakoyiannis *et al.*, 1988; Giessen *et al.*, 1992; Stern, 1992; Gregory *et al.*, 1997; Hudson *et al.,* 1999). In other domestic animals, *C. jejuni* is found in dogs, cats, and horses (Blaser *et al.*, 1980b; Giessen *et al.*, 1992; Stern, 1992). In wild animals within the poultry farm environment, *C. jejuni* is found in rabbits, rats, raccoons, bank voles, hamsters, and mice (Luechtefeld *et al.*, 1980; Kakoyiannis *et al.*, 1988; Giessen *et al.,* 1992; Stern, 1992; Gregory *et al.,* 1997; Hald *et al.*, 2000). In birds, the organism is found in crows, gulls, pigeons, starlings, black birds, sparrows, swans, ducks, cardinals, and black-eyed jungo (Luechtefeld *et al.*, 1980; Kakoyiannis *et al.*, 1988; Stern, 1992; Tauxe, 1992; Gregory *et al.,* 1997; Hudson *et al.*, 1999), and in insects, it is found in beetles and flies including the house fly (Giessen *et al.*, 1992; Stern, 1992; Gregory *et al.*, 1997). Shellfish, laboratory animals, and zoo animals are also documented as reservoirs of *C. jejuni* (Stern, 1992; Misawa *et al.*, 2000). *C. jejuni* therefore, is not host specific.

2.3.2 Environmental reservoirs of *C. jejuni*

In addition to animal reservoirs of *C. jejuni* in the environment, the organism is documented in reviews, as having been isolated from the following inanimate reservoirs: water, litter, poultry feed, broiler house floor, chicken feathers, egg-shells, and boots of farm-workers (Pokamunski *et al.*, 1986; Kakoyiannis *et al.,* 1988; Tauxe, 1992; Gregory *et al.,* 1997; Stern *et al.*, 1997; Hudson *et al.*, 1999). *C. jejuni* appears widely distributed in water. In Norway, Kapperud *et al.*, (1993) isolated *C. jejuni* from a variety of water sources for broilers that included lakes, rivers, brooks, ponds, wells and bore-holes. In New Zealand, Hudson *et al.,* (1999) established the presence of *C. jejuni* in sewage water, a river draining agricultural land, a stream draining a stock sale-yard, and a river downstream from a meat processing plant. *C. jejuni* has also

been isolated from fresh water streams and lakes (Luechtefeld *et al.*, 1980) and a bore-hole supplying water to a poultry house (Pearson *et al.*, 1993). Rollins and Colwell (1986) reported that *C. jejuni* assumes a viable but non-culturable form in water at low temperatures. Therefore *C. jejuni* could be more common in water sources than it is reflected by the documented results of bacteriological cultures.

2.3.3 Public health aspects of *C. jejuni*

The importance of *C. jejuni* to public health became recognized in the 1970s after successful methods for the isolation of the organism from human stools were developed (Norcross *et al.*, 1992; Altekruse *et al.*, 1994). *C. jejuni* has since become the enteric bacterium most commonly isolated from humans with acute diarrhoea exceeding *Salmonella* spp., *Shigella* spp., and *Escherichia coli* particularly in developed countries (Norcross *et al.*, 1992; Friedman *et al.*, 2000). The cost of *Campylobacter* infection to individuals and national health services in developed countries is enormous (Skirrow, 1991). For example, annual cost estimates of poultry–associated human cases of campylobacteriosis in the United States of America (USA), were reported by Bryan and Doyle (1995) to range from US $362 million to US $699 million.

(i) Incidence

As shown in Table 2.3, the incidence of reported human *Campylobacter* infections is high and surveillance in developed countries indicates that it is increasing (Lane, 1993; Friedman *et al.*, 2000). Surveillance studies have revealed a seasonal variation in the incidence with an increase in summer months (Skirrow, 1991; Taylor, 1992b). Also, there is a bimodal age distribution of the incidence with a peak in children under the age of four years and another in young adults of 20 to 30 years of age (Skirrow, 1991; Bryan and Doyle, 1995). The incidence in acquired immunodeficiency syndrome (AIDS) patients was found in one study, to be 39 times higher than the rate among the general public (Sorvillo *et al.*, 1991).

Table 2.3 The incidence of human campylobacteriosis[a]

Country	Incidence	Remarks	Year	Reference[b]
England	55 000 cases/yr	reported	1999	1
England	420 000 cases/yr	estimated	1999	1
Denmark	64 cases/100 000/yr	reported	1998	2
New Zealand	363 cases/100 000/yr	reported	1998	1
USA	21.7 cases/100 000/yr	reported	1998	3
USA	53 cases/100 000/yr	children < 1yr.old	1998	3
USA	2.4 million cases/yr	"true" estimate	1996-98	3
USA	50 to 151 deaths/yr	reported	1996-98	3
USA	23.5 cases/100 000/yr	reported	1996	3
USA	1 to 4 million cases/yr	estimate	1995	7
New Zealand	267 cases/100 000/yr	reported	1993	4
New Zealand	152 cases/100 000/yr	reported	1992	4
New Zealand	123 cases/100 000/yr	reported	1991	4
Australia	32–258 cases/100 000/yr	reported	1991	4
New Zealand	114 cases/100 000/yr	reported	1990	4
England & Wales	85 cases/100 000/yr	reported	1990	5
England & Wales	110 cases/100 000/yr	estimate	1990	5
Yugoslavia	90 cases/100 000/yr	Reported	1989	6
Yugoslavia	2500 cases/100 000/yr	1-4yr old children	1989	6
USA-Los Angels	519 cases/100 000/yr	AIDS patients	1983-87	3
USA- Seattle	60 cases/100 000/yr	Reported	1985	5

a- The incidences are based on surveys carried out at different times and in the countries stated.

b- 1, Frost (2001) 2, Wedderkopp *et al.*, (2000) 3, Friedman *et al.*, (2000) 4, Lane (1993) 5, Skirrow (1991) 6, Popovic-Uroic (1989) 7, Bryan and Doyle (1995).

(ii) Human *C. jejuni* infections

The most common disease caused by *C. jejuni* in humans is gastroenteritis (Altekruse *et al.*, 1994). Following the ingestion of an infective dose of *C. jejuni*, which has been reported to be as low as 500 to 800 bacterial cells (Robinson, 1981; Black *et al.*, 1988), there is a prodromal phase in about 30% of the cases, characterised by fever, headache, myalgia, and malaise (Skirrow and Blaser, 2000). This phase, which is 12 to 48 hours long, is followed by acute diarrhoea accompanied by cramping abdominal pains (Smith, 1995). Other reported symptoms are vomiting, frank blood in the stools, and lethargy (Eberhart-Phillips *et al.*, 1997). Most infections are self-limiting and recovery takes three to four days. However, about 20% of infected individuals have relapses, which prolong the illness (Lane, 1993).

C. jejuni gastroenteritis is the commonest bacterial infection which precedes an acute, inflammatory, demyelinating polyneuritis characterised by paralysis, pain, and muscle wasting called Guillain-Barré Syndrome (GBS) (Hadden and Gregson, 2001). The disease has a fatality rate of 3 to 8 percent (Smith, 1995). A variant of GBS, which is acute and self-limiting, called Miller-Fisher syndrome (MFS), causes paralysis of eye muscles (ophthalmoplegia), muscle inco-ordination (ataxia), loss of reflexes (areflexia), and facial weakness (Ropper, 1992).

Another sequel of *C. jejuni* infection is an acute inflammatory sterile arthritis, called reactive arthritis (Smith, 1995). A subtype of reactive arthritis called Reiter's syndrome, is characterised by arthritis, urethritis, and conjunctivitis (Firestein *et al.*, 1987).

Several extra-intestinal conditions are documented as manifestations of *C. jejuni* infection. They include menigitis, cholecystitis, pancreatitis, hepatitis, appendicitis, glomerulonephritis, osteitis, and septicaemia (Smith, 1995). *C. jejuni* infection has also been reported to cause convulsions in children and delirium in adults (Skirrow and Blaser, 2000). *C. jejuni* has been isolated in human cases of abortion (Gilbert *et al.*, 1981) and premature labour (Miller and Guard, 1982). In AIDS patients, *C. jejuni* infection has been associated with severe recurrent extra-intestinal disease (Angulo and Swerdlow, 1995) including bacteraemia (Guevara *et al.*, 1994). The infection also

shortens survival time in AIDS patients (Sorvillo *et al.*, 1991). According to Bryan and Doyle (1995) the proportion of severe illnesses from *C. jejuni* infections is expected to increase with the growing percentage of the elderly and immuno-compromised individuals in the world population.

(iii) Sources of infection

Investigations by case-control studies and bacteriological culture methods have identified poultry meat, milk, water, and animals as the main sources of *C. jejuni* infection for humans (Taylor, 1992b; Friedman *et al.*, 2000; Frost, 2001).

(a) Poultry

Poultry appears to be the single most common food source for sporadic human *C. jejuni* infection (Friedman *et al.*, 2000; Humphrey *et al.,* 2001). Case-control studies by Harris *et al.*, (1986) and Gilbert (1993) identified the consumption of poultry or chicken as a risk factor associated with over 50% of human sporadic cases of *Campylobacter* enteritis. In particular, the consumption of undercooked chicken has been strongly associated with the transmission of *C. jejuni* (Taylor, 1992b; Eberhart-Phillips *et al.*, 1997). About 62% of poultry products in the USA were reported contaminated with *C. jejuni* (Bryan and Doyle, 1995). In New Zealand 51% of samples from raw ready-for-sale poultry were found contaminated with *C. jejuni* (Gilbert, 1993). According to Bryan and Doyle (1995) each chicken carcass carries up to 10^6 *C. jejuni* bacterial cells. Serovars of *C. jejuni* prevalent in poultry have been found to be the same as those frequently isolated from humans in most developed countries (Friedman *et al.*, 2000). In Sweden and Norway, the seasonal pattern of the incidence of *C. jejuni* in broilers at the time of slaughter, is similar to that of human *Campylobacter* infection, which is consistent with the view that poultry is a source of *C. jejuni* for humans (Friedman *et al.,* 2000). Grados *et al.*, (1983) associated occupational exposure to poultry with an increased risk of human campylobacteriosis.

It has been speculated that commonly, unhygienic practices in the kitchen allow the transfer of *C. jejuni* from poultry to salads and prepared foods and therefore indirect transmission to humans (Bryan and Doyle, 1995; Friedman *et al.*, 2000).

(b) Milk, water, and animals

The consumption of unpasteurized milk or untreated water has been epidemiologically associated with more outbreaks of human campylobacteriosis than other risk factors in developed countries (Blaser *et al.*, 1983; Skirrow, 1991; Frost, 2001). Both milk and water are major sources of *C. jejuni* infection in developing countries (Taylor, 1992b). It has been speculated that water and milk are sources of *C. jejuni* for a proportion of human cases of gastroenteritis associated with foreign travel, so- called "traveller's diarrhoea" (Eberhart-Phillips *et al.*, 1997).

Case-control studies have identified contact with household pets as a risk for *Campylobacter* infection (Harris *et al.*, 1986; Brieseman, 1990; Frost, 2001). In particular, contact with puppies or kittens with diarrhoea, has been found to be a risk factor (Fleming, 1983; Eberhart-Phillips *et al.*, 1997).

(iv) Anti-microbial resistance

C. jejuni is capable of developing resistance to anti-microbial drugs. Of the commonly used antibiotics, resistance of *C. jejuni* isolates is documented as having been demonstrated against trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, ciprofloxacin, erythromycin, clindamycin, and chloramphenicol (Friedman *et al.*, 2000). Multi-drug resistance is also documented (Taylor, 1992a; Tenover *et al.*, 1992). A marked increase in rates of quinolone resistance among human *Campylobacter* isolates was observed in Spain after the introduction of fluoroquinolones into the country for use in poultry (Saenz *et al.*, 2000; Smith *et al.*, 2000).

(v) Control measures

With respect to poultry, *C. jejuni* contamination remains unavoidable (Norcross *et al.*, 1992: Humphrey *et al.*, 2001). Irradiation is recognised as an effective control measure (Norcross *et al.*, 1992; Friedman *et al.*, 2000). However, this method of preservation is yet to be widely accepted by the public. Irradiation has been

recommended for food intended for individuals at risk of serious infection such as the elderly and patients with cancer or AIDS (Altekruse *et al.*, 1994).

Food preparation is a major critical point for the control of *C. jejuni* (Norcross *et al.*, 1992). According to Altekruse *et al.*, (1994) public education on handling of foods, particularly poultry, can reduce human *C. jejuni* infection. Based on risk factors identified by case-control studies, Altekruse *et al.*, (1994) have emphasised the need for adequate cooking of poultry and poultry products, care not to cross-contaminate other foods in the kitchen, and avoidance of the consumption of raw milk and untreated water.

Contact with pets, in particular diarrhoeic puppies or kittens should be avoided by children, the elderly, and individuals with debilitating disease (Altekruse *et al.*, 1994). In the case of such contact, hands should be washed with soap and dried shortly afterwards (Altekruse *et al.*, 1994).

2.3.4 Transmission of *C. jejuni* to broilers

Campylobacter species are endemic in commercial broiler flocks. Surveys of flock prevalence have shown that at the time of slaughter, 76% of the flocks were infected in the United Kingdom (Humphrey *et al.*, 1993), 57% in the Netherlands (Giessen *et al.*, 1996), 27% in Sweden (Berndtson *et al.*, 1996), 18% in Norway (Kapperud *et al.*, 1993), and 52% in Denmark (Hald *et al.*, 2000). Due to the wide distribution of *C. jejuni* in both animate and inanimate reservoirs, the sources of infection for broilers remain unclear (Hald *et al.*, 2000; Shreeve *et al.*, 2000; Pattison, 2001).

(i) Vertical transmission

Newly hatched chicks from breeder flocks have consistently been found to be *Campylobacter* free (Doyle, 1984; Genigeorgis *et al.*, 1986; Annan-Prah and Janc, 1988; Jones *et al.*, 1991). In addition, it is uncommon to culture *Campylobacter* spp. from broiler flocks in their first two weeks of life (Kazwala *et al.*, 1990; Jacobs-Reitsma *et al.*, 1995; Gregory *et al.*, 1997). It has also been found that *C. jejuni* does not penetrate the egg-shell (Neill *et al.*, 1985; Clark and Bueshkens, 1988). Therefore,

it is widely accepted that vertical transmission is highly unlikely under natural conditions (Annan-Prah and Janc, 1988; Jones *et al.,* 1991; Giessen *et al.,* 1992).

Shanker *et al.*, (1986) experimentally demonstrated vertical transmission by injecting eggs with *C. jejuni* and recovering the organism from the intestines of chicks when the eggs hatched. Also Lindblom *et al.,* (1986) isolated *C. jejuni* from newly hatched chicks that were in a protected laboratory environment. After observing the introduction of *C. jejuni* serotypes in broiler flocks, that were similar to the serotypes isolated from the parent flocks, Pearson *et al.,* (1996) speculated that the infection was transmitted vertically through the hatcheries supplying the farms with day-old chicks. Recent evidence based on molecular typing techniques also suggests that campylobacters could pass from one generation of broilers to the next via the egg (Stern *et al.,* 2000).

(ii) Horizontal transmission

In contrast to vertical transmission, there is a general agreement that horizontal transmission of campylobacters occurs from reservoirs in the environment to broilers (Kazwala *et al.,* 1990; Giessen *et al.*, 1992; Stern, 1992; Hald *et al.*, 2000). When *C. jejuni* is introduced to *Campylobacter* free chickens by an experimentally infected chicken, the organism spreads to the birds (Lindblom *et al.*, 1986; Kazwala *et al.*, 1990; Shanker *et al.,* 1990). Epidemiological studies have been carried out to identify risk factors for the introduction of *C. jejuni* to broilers based on the assumption of horizontal transmission (Jacobs-Reitsma *et al.,* 1995; Gregory *et al.*, 1997; Hald *et al.*, 2000; Wedderkopp *et al.*, 2000).

(a) Hatchery

The isolation of *C. jejuni* from the environment of commercial hatcheries is reported to be uncommon (Oosterom *et al.*, 1983; Lindblom *et al.*, 1986; Annan-Prah and Janc, 1988; Humphrey *et al.*, 1993). Pearson *et al.*, (1996) observed a tendency for chicks from one of two hatcheries to have a higher frequency of *C. jejuni* infection and they speculated that the hatchery could be the source of infection. Clark and Bueshkens (1988) demonstrated experimentally that human and poultry derived strains of *C.*

jejuni can spread horizontally among broiler chicks held in incubators and shipping boxes. In their experiment, up to 70% of chicks from an incubator in which an infected chick was placed, were found to have *C. jejuni* in their intestinal tract.

(b) The poultry shed

Poultry flocks that are colonised by *C. jejuni* contaminate the environment of the poultry shed which can potentially serve as a source of infection for subsequent broiler flocks (Shanker *et al.*, 1990). A short interval between successive flocks in a shed has been identified as a risk factor associated with the introduction of campylobacters to broilers (Hald *et al.*, 2000; Wedderkopp *et al.,* 2000). Conversely, periods longer than two weeks between successive flocks were associated with fewer *Campyloacter* positive flocks (Berndtson *et al.*, 1996; Hald *et al.*, 2000).

A single *C. jejuni* serotype was observed to persist in successive flocks reared in the same house and the disinfection of the house between flocks was reported to be inadequate (Giessen *et al.,* 1992). Studies have often been inconclusive with respect to the poultry house being either the source of *C. jejuni* or the route for horizontal transmission of the organism to successive broiler flocks (Smitherman *et al.*, 1984; Pearson *et al.*, 1993; Jacobs-Reitsma *et al.*, 1995). According to Gregory *et al.*, (1997) whether a broiler house is new or had previously been used does not affect the transmission of campylobacters.

(c) Water, litter, and feed

Water supplied unchlorinated has been identified as a source of campylobacters for broilers (Kapperud *et al.*, 1993; Pearson *et al.*, 1993). Shanker *et al.*, (1990) challenged broiler chicks with water experimentally contaminated with *C. jejuni* and demonstrated that chicks are rapidly colonised by the organism. Hald *et al.*, (2000) identified contaminated water as a risk factor for occurrence of campylobacters in broilers. Within the poultry house, however, water has consistently been found negative for campylobacters prior to the infection of the chickens (Smitherman *et al.*, 1984; Genigeorgis *et al.,* 1986; Gregory *et al.,* 1997).

Litter and feed have often been found negative for campylobacters (Smitherman *et al.,* 1984; Jones *et al.*, 1991; Humphrey *et al.*, 1993; Gregory *et al.*, 1997), which indicates that they are an unlikely source of *C. jejuni.* Experimentally contaminated litter (Montrose *et al.*, 1985) and feed (Al-Obaidi, 1988 cited by Evans, 1992) have been reported to transmit *C. jejuni* to broiler chicks. The susceptibility of *Campylobacter jejuni* to drying and atmospheric oxygen tension is thought to preclude its survival in litter and feed (Pearson *et al.*, 1993; Genigeorgis *et al.*, 1986). In feed, *C. jejuni* is thought to be killed by the drying and pelleting process (Evans, 1992) and by antibiotics, which are often included in poultry feed (Lindblom *et al.*, 1986).

(d) Footwear, transport vehicles, and equipment

The role of footwear, transport vehicles such as trucks, and other equipment such as crates and shipping boxes in the transmission of *C. jejuni* to broilers, has been investigated and they have been speculated to be vectors that introduce the infection to susceptible birds from an unknown environmental source (Lindblom *et al.,* 1986; Annan-Prah and Janc, 1988; Evans, 1992; Giessen *et al.*, 1998). Hald *et al.,* (2000) found the lack of disinfection of workers' boots at the entrance of a broiler house to be associated with occurrence of *C. jejuni* in broilers. Using random amplified polymorphic DNA (RAPD) typing technique, Giessen *et al.,* (1998) managed to link an isolate of *C. jejuni* from the boots of a farmer to identical isolates concurrently cultured from broilers and cattle on the same farm. In the same study, *C jejuni* was isolated from slaughterhouse crates and a lorry used during partial depopulation of broiler houses.

A management practice, referred to as partial depopulation, where broiler flocks are 'thinned' at about five weeks of age, when they first reach slaughter age, has been observed in Sweden, Denmark, the Netherlands, and the United Kingdom (Berndtson *et al.,* 1996; Jacobs-Reitsma, 1997; Hald *et al.*, 2000; Wedderkopp *et al.*, 2000; Pattison, 2001). During partial depopulation, catching crews and their equipment such as slaughterhouse crates, are reported to break the hygiene barrier when they enter broiler houses (Berndtson *et al.*, 1996; Jacobs-Reitsma, 1997). The practice of thinning flocks or dividing flocks into two or more groups for slaughter at different

ages, has been associated with the introduction of *C. jejuni* to broilers (Berndtson *et al.,* 1996; Hald *et al*., 2000; Wedderkopp *et al*., 2000; Pattison, 2001)

(e) Animals on farm premises

There is a paucity of reported epidemiological evidence confirming the transmission of *C. jejuni* from animal reservoirs to broiler chickens. Farm animals including sheep, cattle, and pigs are known to carry campylobacters (Annan-Prah and Janc, 1988; Jacobs-Reitsma *et al*., 1994) and campylobacters in environmental samples such as water, can be considered evidence of faecal contamination from animals (Kapperud *et al*., 1993; Jones, 2001). The presence of livestock other than broilers on poultry farm premises has been identified as a risk factor associated with increased occurrence *Campylobacter* spp. in broiler flocks (Giessen *et al.,* 1996; Hald *et al*., 2000). Pig production in particular, was found to be associated with the presence of campylobacters in broilers by Kapperud *et al*., (1993). In contrast, Jacobs-Reitsma *et al.,* (1994) found no statistically significant association between keeping pigs on a farm and the presence of campylobacters in broiler flocks.

Giessen *et al.,* (1998) isolated *C. jejuni* of an identical RAPD-type in cattle and several successive broiler flocks on the same farm and they argued that cattle were the source of infection for the broilers. Gregory *et al*., (1997) reported isolating *C. jejuni* from cattle and wild birds in the vicinity of broiler houses prior to the broilers becoming colonised by the organism. Their isolates were characterised by Stern *et al*., (1997) using flagellin A (*flaA*) gene restriction fragment length polymorphsm (RFLP) and three *flaA*-types from within the broiler houses, were found to be indistinguishable from those isolated from animals outside the boiler houses.

Giessen *et al*., (1998) isolated *C. jejuni* from insects and the RAPD patterns of the isolates were identical to those of isolates from broilers on the farm indicating that insects could have served as vehicles of transmission in the broilers. Rosef and Kapperud (1983) reported a high prevalence of *C. jejuni* (50.7%) in 146 flies captured on a chicken farm. Shane *et al.,* (1985) demonstrated experimentally that house flies (*Musca domestica*) could transmit *C. jejuni* to broilers.

Although established as potential sources of *C. jejuni* for broilers, sheep, horses, wild birds, dogs, cats, rodents, and humans, are yet to be epidemiologically proven as sources of *C. jejuni* infection for broilers.

2.4 PREVENTION AND CONTROL OF *C. JEJUNI* IN POULTRY

The main objective of intervention measures during the rearing period of poultry is to prevent colonization of the birds by campylobacters. If successful, the benefit would be the production of *Campylobacter* free poultry or a significantly decreased *Campylobacter* flock prevalence both of which would be expected to reduce poultry associated human campylobacter enteritis (Kapperud *et al.*, 1993; Lane, 1993; Giessen *et al.*, 1998; Evans and Sayers, 2000; Hald *et al.*, 2000; Newell and Wagenaar, 2000; Gibbens *et al.*, 2001). Measures taken within the slaughterhouse to reduce *Campylobacter* contamination of poultry carcasses are thought to be ineffective hence the high prevalence of *C. jejuni* in poultry products and the associated high incidence of human campylobacteriosis (Berndtson *et al.*, 1996; Evans and Sayers, 2000; Gibbens *et al.*, 2001).

Reported intervention strategies that can be applied in commercial poultry farms involve mainly sanitation and biosecurity (Giessen *et al.*, 1992, 1996; White *et al.*, 1997; Giessen *et al.*, 1998). Other strategies such as biological control procedures, immunization, and medication, are still unavailable to farmers (Altekruse *et al.*, 1994; White *et al.*, 1997; Stern *et al.*, 2000).

2.4.1 Sanitation and biosecurity.

The following measures have been found by epidemiological studies to be successful in either preventing or delaying the colonization of broilers by campylobacters:

- Provision of clean, chlorinated water supply (Kapperud *et al.*, 1993; Pearson *et al.*, 1993; Hald *et al.*, 2000; Gibbens *et al.*, 2001).

- Thorough cleaning of the broiler house followed by disinfection before re-stocking (Shanker *et al.,* 1990; Giessen *et al.*, 1992; Kapperud *et al.*, 1993; Giessen *et al.*, 1996, 1998; Gibbens *et al.*, 2001). Cracks on the floor and the walls of the house should be repaired to improve the effectiveness of cleaning and disinfection (Giessen *et al.*, 1992, 1998).

- An empty period for the broiler house, of at least 14 days between successive broiler flocks (Kapperud *et al.*, 1993; Giessen *et al.*, 1996; Hald *et al.*, 2000).

- A hygiene barrier in the entrance room to the broiler house (Giessen *et al.,* 1998). Hygiene routines to be followed at the barrier before entering the broiler house should include dipping boots in a footbath containing a disinfectant, changing protective clothing and footwear to those dedicated for use within the broiler house only, and washing and drying of hands (Humphrey *et al.*, 1993; Kapperud *et al.*, 1993; Giessen *et al.*, 1996, 1998; Hald *et al.*, 2000). The farmer should be more vigilant at the hygiene barrier after contact with other animals on the farm particularly pigs, cattle, and poultry other than broiler chickens (Kapperud *et al.*, 1993).

- The prompt removal of dead birds from the broiler house and site (Jones *et al.*, 1991; Evans and Sayers, 2000).

- The control of vermin on the farm including rodents, wild birds, and insects (Evans and Sayers, 2000; Newell and Wagenaar, 2000; Corry and Atabay, 2001). The broiler house should be vermin proof (Giessen *et al.*, 1992, 1996, 1998). Also, the house should be inaccessible to domestic and feral animals, pets, children, and uninvited visitors (White *et al.*, 1997; Corry and Atabay, 2001).

- Avoiding the practice of 'thinning' or harvesting part of the flock early (Hald *et al.*, 2000).

The chlorination of water supply and the cleaning and disinfection of the drinking system was found to reduce the prevalence of *C. jejuni* in broilers from 81% to 7% in a farm where untreated borehole water was suspected to be the source of infection (Pearson *et al.,* 1993). In another study, the introduction of hygiene measures was found to decrease the prevalence of *Campylobacter* infection in broilers from 66% to 22% on one farm and 100% to 42% on another (Giessen *et al.*, 1998). More recently, Gibbens *et al.*, (2001) reported a 50% reduction in *Campylobacter* colonization of broilers following the introduction of strict hygiene and biosecurity measures in selected commercial farms in Great Britain. Several studies have also demonstrated that broiler flocks can be raised *Campylobacter* free to slaughter age by applying strict hygiene and biosecurity measures (Lindblom *et al.,* 1986; Shanker *et al.,* 1990; Giessen *et al.*, 1996, 1998; Shreeve *et al.*, 2000; Gibbens *et al.*, 2001).

The limitation with hygiene and biosecurity measures is that compliance with them on a routine basis is difficult (Giessen *et al.*, 1998; Gibbens *et al.*, 2001). Also, the measures do not eliminate the risk of re-introduction of *C. jejuni* from environmental sources (Giessen *et al.*, 1998). It has been found that even the most stringent biosecurity measures do not prevent the colonization of broilers with campylobacters (Shreeve *et al.*, 2000; Pattison, 2001).

2.4.2 Biological control

Competitive exclusion (CE) microflora, when administered to broiler chicks prior to challenge with *C. jejuni*, were reported to sharply reduce colonization with *C. jejuni* (Söejardi *et al.*, 1982). Stern (1994) also demonstrated experimentally that treatment of chicks with intestinal commensal bacteria derived from broilers could reduce *C. jejuni* colonization. Contrasting findings have also been reported where CE microflora were found to have no effect on the colonization rate of broilers by *C. jejuni* (Stern *et al.,* 1988). The control of *C. jejuni* infection of broilers by CE microflora is still under investigation and it is currently unavailable to farmers (White *et al.*, 1997). Another potential control measure is the rearing of broiler chickens that are resistant to colonization by *C. jejuni* (Stern *et al.*, 1990a). This measure is perceived as a long-term goal as the selection of *Campylobacter* resistant chicken lines requires considerable time.

2.4.3 Immunization

Experimental studies have shown that administration of an oral vaccine could potentially prevent colonization of broilers by *C. jejuni* (Myszewski and Stern, 1990; Stern *et al.,* 1990b). Anti-campylobacter secretory immunoglobulin A (sIgA) has been shown to neutralize *C. jejuni* colonization factors resulting in a ten–fold reduction in the colonization potential of the organism (Stern and Meinersmann, 1989). According to Stern *et al.*, (1990b) the success of immunization depends on the development of techniques to increase the anti-campylobacter sIgA response in the birds.

2.4.4 Medication

It is uncommon to administer antibiotics to broilers for the control of *C. jejuni.* However, antibiotics usually included in feed to control other bacterial pathogens could diminish *C. jejuni* in the intestinal tract of broilers (Lindblom *et al.*, 1986). Refregier-Petton *et al.*, (2001) found that antibiotic treatment of broiler flocks for specific diseases was associated with a decreased risk of *Campylobacter* colonization of the birds. However, the treatment of broilers with antibiotics is undesirable as it may cause the emergence of antibiotic-resistant *Campylobacter* strains (Aarestrup and Wegener, 1999; Saenz *et al.*, 2000).

2.5 AIMS OF THE THESIS

(i) To investigate the prevalence of *Campylobacter jejuni* on the footwear of poultry slaughterhouse personnel and the fomites from the slaughterhouse used during the depopulation of broiler flocks.

(ii) To assess the effect of the cleaning carried out at the slaughterhouse on the level of *C. jejuni* contamination found on the footwear of slaughterhouse personnel and fomites.

(iii) To demonstrate whether or not, boots can transmit *C. jejuni* to susceptible broiler chickens.

(iv) To determine the transmission rate of *C. jejuni* in broiler chickens.

CHAPTER 3

3. GENERAL MATERIALS AND METHODS

3.1 SAMPLING

Samples were taken with cotton tipped swabs (Fort Richards, Auckland, New Zealand). When sampling fomites, as described in chapter 4, swabs were moistened in buffered peptone water before sampling. During the experiment with broiler chickens (Chapter 5), cloacal swab samples were taken.

3.2 ISOLATION OF *C. JEJUNI*

3.2.1 Selective enrichment

Swab samples were immediately inoculated into Bolton's broth for selective enrichment. Within two hours, the broths were incubated at 42°C under microaerophilic conditions for 48 hours. The samples were incubated in an anaerobic jar in which Campy Gen^{Tm} gas generating sachet (Oxoid; CN 25, Hampshire, England), had been placed. The lids of the containers were loosened during incubation. An alternative to the gas generating system was the use of a 42°C triple gas incubator (Jouan^{Tm}, Saint-Herblain, France). An atmosphere of 5% O_2, 10% CO_2 and 85% N_2 was attained in the incubator by infusion of the gases from gas cylinders.

3.2.2 Culturing on solid selective media

A sterile swab was used to inoculate Bolton's broth onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA; Oxoid CM739 and SR155E, Hampshire, England). The plates were incubated at 37°C under microaerophilic conditions for 48 hrs.

3.2.3 Sub-culturing on non-selective media

Single colonies with characteristic appearance of *Campylobacter jejuni*, were plated on Tryptic Soy Agar (TSA) plates to obtain pure cultures. The TSA plates were incubated at 42°C under microaerophilic conditions for 48 hrs.

3.3 PRESUMPTIVE IDENTIFICATION

3.3.1 Colony morphology

The identification of *Campylobacter jejuni* was initiated with the examination of colony morphology on mCCDA plates. *Campylobacter* spp. colonies on mCCDA were typically grey, convex with irregular margins, glistening, spreading along streak marks or forming a "lawn" over the surface of the medium. The colonies on TSA were characteristically small, 1-2 mm in diameter although swarming growths were also found particularly in moist media. Growth at 37°C and 42°C was considered supportive evidence that the organisms were thermophilic.

3.3.2 Sensitivity to nalidixic acid and cephalothin

A single colony from mCCDA or TSA plate was inoculated with a sterile straight wire onto a TSA plate. A sterile swab was used to spread the inoculum evenly across the surface of the plate. A 30 µg nalidixic acid disc (NA; Oxoid, Hampshire, England) was placed on one side of the plate. A 30 µg cephalothin disc (KF; Oxoid, Hampshire, England) was placed on the opposite side of the plate. The plate was incubated at 42°C for 48 hrs under microaerophilic conditions. A clear zone of inhibition around a disc indicated that the isolate was sensitive to the antibiotic in the disc while growth to the margin of the disc indicated resistance.

3.3.3 **Hippurate hydrolysis test** (Skirrow and Benjamin, 1980a)

A loopful of a 48-hour growth culture from a TSA plate was emulsified in a test tube with 0.4 ml distilled water and one hippurate hydrolysis disc (BBL^{Tm} TAXO^{Tm}; Becton Dickinson and Co., St Louis, USA). The tube was incubated at 37°C in a water bath or an incubator for two hours. Then, 0.2 ml (5 drops) of ninhydrin reagent (BBL®; Becton Dickinson and Co., Cockeysville, USA) was added. The tube was shaken, incubated for a further 15 minutes and observed for results. A positive result was indicated by the presence of a deep purple colour. *Campylobacter jejuni* subsp. *jejuni* (NCTC 11351) was used as a positive control. The negative control organism was *Campylobacter coli* (NCTC 11366). Plate 3.1 shows the colour of a positive hippurate hydrolysis test represented by NCTC 11351 and *C. jejuni* 45 and a negative test represented by NCTC 11366.

3.3.4 Catalase test

A loopful of growth from a TSA plate was placed on a microscope slide. A single drop of 3% H_2O_2 was added to the growth and observed for bubbles. The development of bubbles was taken to indicate that the bacteria were catalase positive.

3.3.5 Oxidase test

A colony was transferred with a sterile loop onto a moistened oxidase impregnated paper strip (Medvet Science, Adelaide, South Australia). The appearance of a purple colour where the bacteria was in touch with the paper, within 10 seconds, indicated a positive result.

3.3.6 Microscopic examination

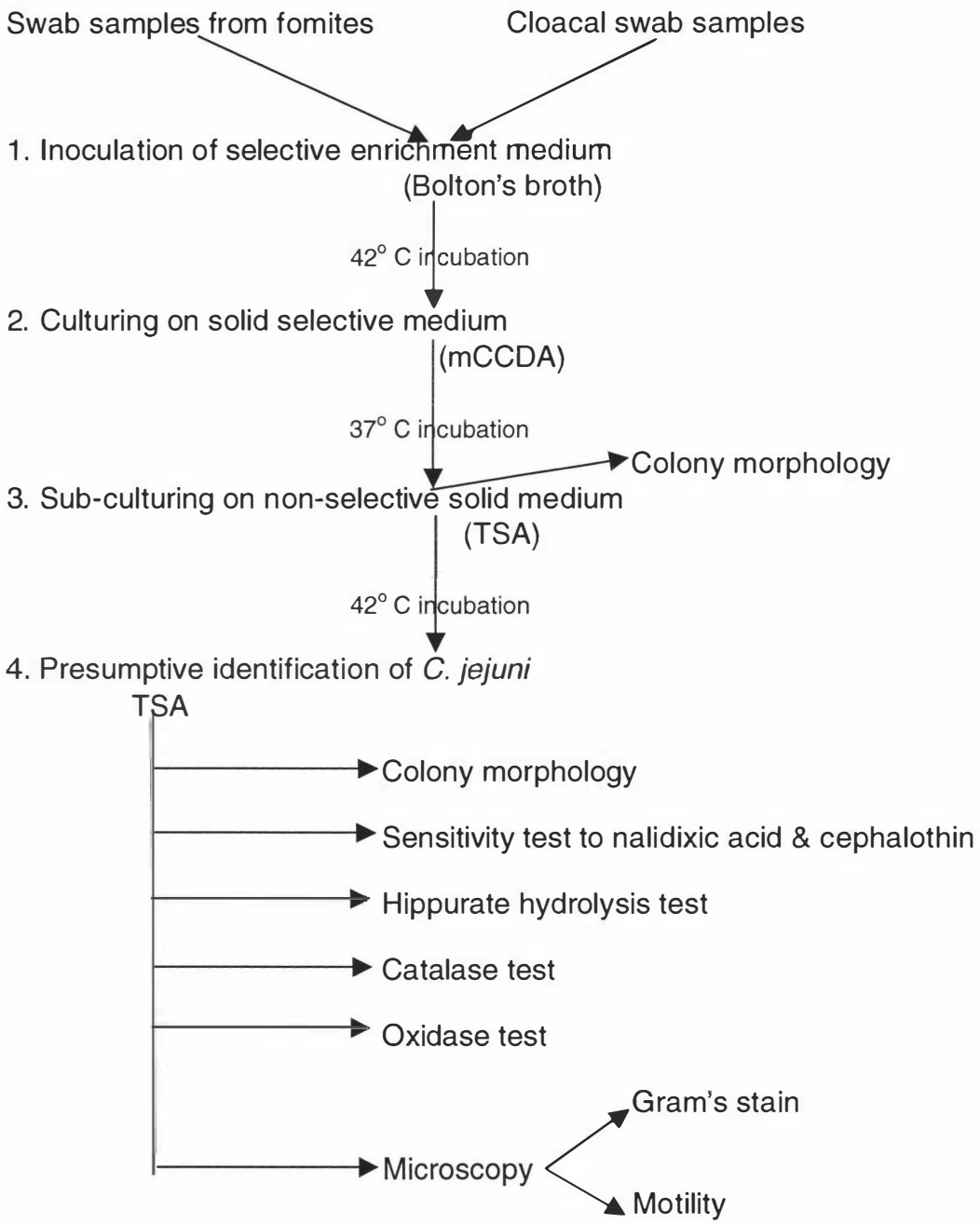
A loopful of bacterial growth was emulsified into a drop of saline water on a microscope slide. After air-drying and heat fixing, the slide was stained by Gram's method and examined under 100x objective of a light microscope. The bacteria were presumed campylobacters if they were Gram negative and, depending on the age of the culture, appeared in one of the following forms; curved or spiral rods, comma-

shaped, gull-wing shaped, or coccoid. Old cultures turned to have more of the coccoid form of the bacteria. Plate 3.2 shows a microscopic appearance of organisms that were presumed to be *Campylobacter* species. A wet preparation of each isolate was examined with a dark field microscope to observe motility and morphology.

3.4 PRESERVATION OF CULTURES

Isolates that were identified as *C. jejuni* were sub-cultured on TSA plates and examined for purity. Fourty-eight hour pure growths from these plates were transferred with sterile swabs into 3 ml aliquots of 15% glycerol broths contained in bijoux bottles. Using sterile 5-inch glass pipettes, the bacterial suspension of each isolate, in glycerol broth, was mixed and then transferred to 2 ml vials (Cryovials®; Greiner Labortechnick, Frickenhausen, Germany). The vials were filled to the calibration mark and stored at $-70^{o}C$ until required.

Figure 3.1 Steps followed in the isolation and identification of *C. jejuni*



All media were incubated under microaerophilic conditions for 48 hrs

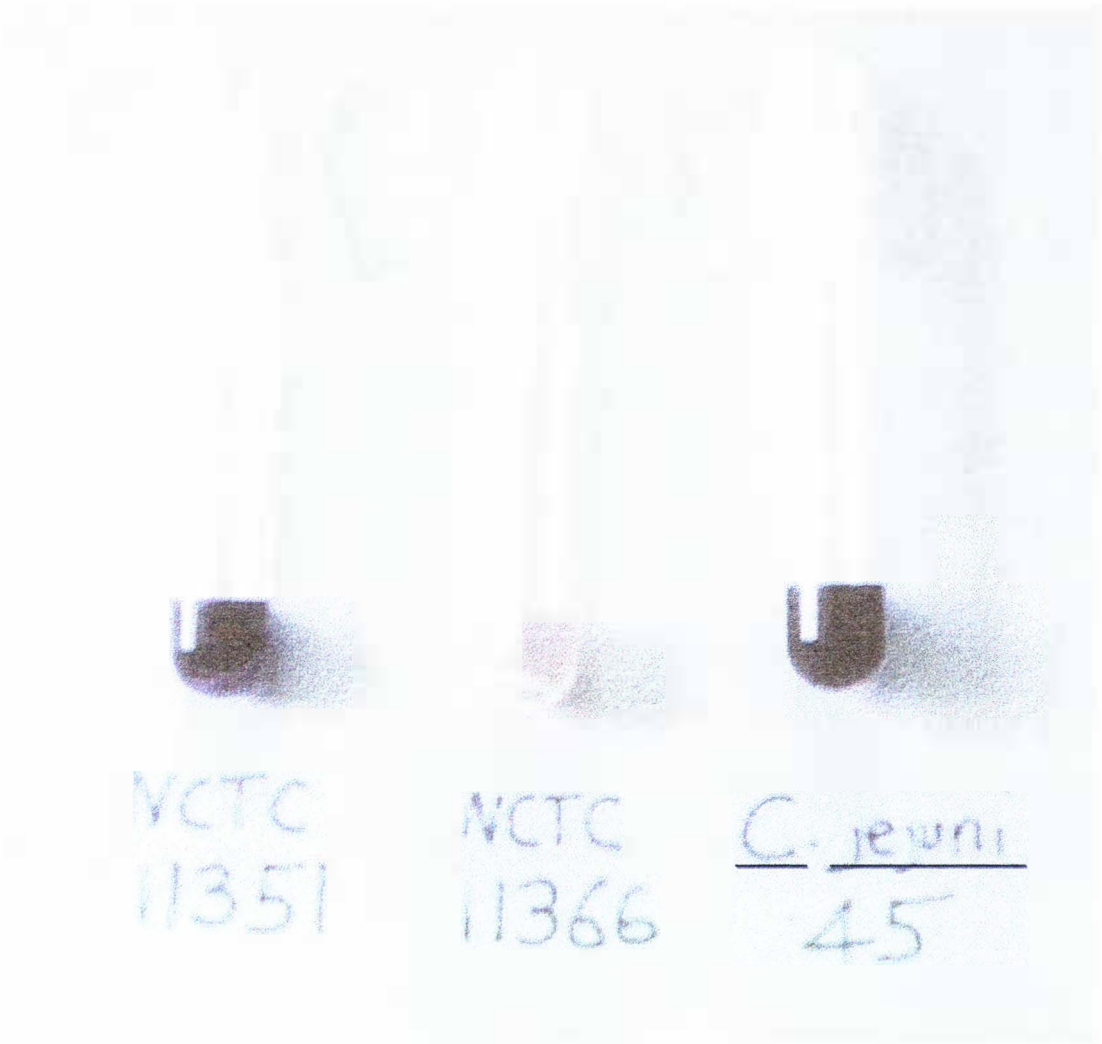


Plate 3.1 The test for hippurate hydrolysis. NCTC 11351 is a positive control. NCTC 11366 is a negative control. *C. jejuni* 45 is an isolate from a broiler chicken.

Plate 3.2 A Gram stain showing morphology of bacteria presumed to be *Campylobacter* species

CHAPTER 4

4. POTENTIAL SOURCES FOR TRANSMISSION OF *C. JEJUNI* TO BROILER CHICKRNS

4.1 INTRODUCTION

Poultry meat has been established as an important source of human infection with both *Campylobacter jejuni* and *Campylobacter coli* (Skirrow, 1991; Pearson *et al.*, 1996). As a result, there is a general consensus that a significant reduction in human infections can be achieved by reducing *Campylobacter* infection in broiler flocks (Lindblom *et al.*, 1986; Evans, 1992; Giessen *et al.*, 1998). Many investigations have therefore, been carried out to identify the sources of *C. jejuni* for broilers.

Findings by Doyle (1984), Lindblom *et al.*, (1986), and Pearson *et al.*, (1996) indicate that vertical transmission of *C. jejuni* from the parent flock to chicks is highly unlikely under natural conditions. On the other hand, horizontal transmission is known to occur and important potential sources include the hatchery, poultry sheds, water, litter, feed, fauna, and footwear (Clark and Bueschkens, 1988; Kazwala *et al.*, 1990; Shanker *et al.*, 1990; Kazwala *et al.*, 1992; Pearson *et al.*, 1993). A combination of intervention measures targeting the common sources of infection and improved biosecurity, have been reported to prevent or reduce infection in broilers (Kazwala *et al.*, 1992; Giessen *et al.*, 1998). Jacobs-Reitsma *et al.*, (1995) observed that *Campylobacter* positive and negative flocks were present at the same time on the same farm, even up to slaughter age. This observation led them to conclude that *Campylobacter* free rearing of broilers is viable under commercial farm conditions.

A significant epidemiological feature of *Campylobacter* infection in broilers is that the organism spreads rapidly following introduction (Stern, 1992; Cawthraw *et al.*, 1996). Studies in which broiler flocks were monitored for *Campylobacter* colonization by

taking cloacal swab samples and faecal droppings have demonstrated that virtually all the chickens become positive within one week of exposure (Smitherman *et al.*, 1984). Experimental studies indicate that the spread from an infected bird to susceptible birds occurs within 72 hours (Clark and Bueschkens, 1988; Shanker *et al.*, 1990) and that in large flocks of 20,000 birds, the spread could be logarithmic in nature (Montrose *et al.*, 1985). It is envisaged that *Campylobacter jejuni* could be introduced to *Campylobacter* free flocks at point of slaughter by equipment and personnel during the catching of chickens. The breach in biosecurity that is purported to occur during the catching operation would increase the risk of *C. jejuni* spread to other flocks on the farm. In view of these risk factors, an investigation was carried out to determine if the footwear of personnel who catch and transport chickens to a slaughterhouse, and the trucks, tractors, forklifts, pallets and crates that they use in the exercise, were contaminated with *C. jejuni*. In addition, it was intended to determine the prevalence of *C. jejuni* on all these fomites. Another objective was to assess if the level of contamination of the fomites with *C. jejuni* changes with time during the day.

4.2 MATERIALS AND METHODS

4.2.1 Sampling, isolation and identification of *C. jejuni*

All samples were taken in the premises of a single commercial poultry processing plant. Pallets, crates, truck beds, truck wheels and the drivers' boots were sampled before each depopulation trip just after washing. Catchers' boots were sampled in the morning before the catchers left for work. The tractors made one trip a day and they were sampled just before they left. Forklifts sometimes made more than one trip a day in which case they were sampled more than once.

A swab method was used to take all the samples. A 5 cm^2 metal template was pressed against the surface to be sampled. A cotton tipped swab was moistened with buffered peptone water and rubbed up and down and across the entire area exposed by the template. A second dry swab was rubbed over the surface in the same manner. The

swabs were broken below the handling area so that the cotton tips fell into a universal bottle containing 15 ml of Bolton's enrichment broth.

Each pallet was sampled from the floor of one of its rectangular holes used by the forklift. The hole on the right was sampled each time. Each crate was turned upside down and sampled on the bottom left corner. For each truck, the bed was divided, by inspection, into eight equal sections numbered one to eight. A random number table was used to select four sites. All sections were sampled on the bottom corner away from the center of the truck. A random number table was used to select eight wheels from either 20 or 22 truck wheels. A flat surface on each of the selected wheels, which contacts the soil, was sampled. Three of the four tractor wheels were randomly selected and sampled. All the three forklift wheels were sampled. The surfaces sampled on the tractor and forklift wheels were the same as for the trucks. Samples from the boots of the drivers and catchers were taken from the bottom surface of the heel sole. Only the left boots were sampled.

Overall, there were six days of sampling. On each of the days, six consecutive pooled swab samples representing six consecutive depopulation trips were taken from each of the following: pallets, crates, truck bed, truck wheels, and drivers' boots (Table 4.1). The samples were taken in hourly periods between 0530 hours and 1230 hours. One sample from drivers' boots was missed on the first day of sampling. It was realized when the sampling had already started that tractors were also a potential source for transmission of *C. jejuni* and they were sampled from the third to the sixth day.

The isolation and identification of *Campylobacter jejuni* was carried out using the method described in chapter 3 and illustrated in Figure 3.1

Table 4.1 The number of samples taken from slaughterhouse fomites for investigation of *C. jejuni*

Day of sampling		Pallets	Crates	Truck bed	Truck wheels	Drivers' boots	Catchers' boots	Forklift wheels	Tractor wheels
1	A	18	72	6(24)	48	5	6	9	ND
	B	36	144	48	96	10	12	18	ND
	C	6	6	6	6	5	1	3	ND
2	A	18	72	6(24)	48	6	6	12	ND
	B	36	144	48	96	12	12	24	ND
	C	6	6	6	6	6	1	4	ND
3	A	18	72	6(24)	48	6	6	9	6
	B	36	144	48	96	12	12	18	12
	C	6	6	6	6	6	1	3	2
4	A	18	72	6(24)	48	6	6	6	6
	B	36	144	48	96	12	12	12	12
	C	6	6	6	6	6	1	2	2
5	A	18	72	6(24)	48	6	6	6	6
	B	36	144	48	96	12	12	12	12
	C	6	6	6	6	6	1	2	2
6	A	18	72	6(24)	48	6	6	6	6
	B	36	144	48	96	12	12	12	12
	C	6	6	6	6	6	1	2	2
Total no. sampled		**108**	**432**	**36 (144)**	**288**	**35**	**36**	**48**	**24**
Total of swabs		**216**	**864**	**288**	**576**	**70**	**72**	**96**	**48**
Total of pooled samples		**36**	**36**	**36**	**36**	**35**	**6**	**16**	**8**

A- No. of fomites sampled.
B- No. of swabs taken; half of which were wet and the other half dry.
C- No. of pooled samples.
ND- not done.
( ) – the number in brackets is that of sites on the truck bed that were sampled.

4.3 RESULTS

Campylobacter jejuni was isolated from all sampled sites except the tractor wheels (Table 4.2). The pallets had the highest prevalence. Catchers' boots, drivers' boots, crates, and truck wheels, each had a prevalence of over 50%. Pallets, crates, drivers' boots, catchers' boots, forklift wheels, and tractor wheels enter the broiler shed during depopulation and are a potential source of direct transmission of *C. jejuni*. These fomites, when excluding tractor wheels, had an average percentage positive of 57.1. As discussed bellow, the negative result for tractor wheels is thought to misrepresent their role as potential sources for *C. jejuni*.

The percentage of positive samples was lowest for batch number 1, and highest for batch number 5 (Table 4.3). In two batches of samples, the 5th batch on day 3 and the 4th batch on day 4, *C. jejuni* was isolated from all the five potential sources of infection. In one batch, the 3rd batch on day 6, all the potential sources were negative for *C. jejuni* (Table 4.3). In general, more *C. jejuni* positives were found in the samples taken subsequent to the first sample (Figure 4.1). However, there was no statistically significant difference ($P>0.05$) between the percentage positive of the first batch of samples and that of any subsequent batches.

Table 4.2 Isolation rates of *C. jejuni* from slaughterhouse fomites

Source	n	No.of positives	% positives	Relative risk
Pallets	36	27	75	2.4
Crates	36	21	58.33	1.9
Truck bed	36	17	47.22	1.5
Truck wheels	36	18	50	1.6
Drivers' boots	35	19	54.29	1.7
Catchers' boots	6	4	66.67	2.1
Forklift wheels	16	5	31.25	1
Tractor wheels	8	0	0	
Totals	**209**	**111**	**53.11**	

Table 4.3 Isolation rates of *C. jejuni* from slaughterhouse fomites at different time periods during the day[a]

Batch no. (Sampling period)	Number of positive samples n=5						Sum of positives	% positives
	Day1	Day2	Day3	Day4	Day5	Day6	n=30	
1(0530-0630)	3[b]	2	3	1	2	2	13[c]	44.83
2(0630-0730)	4	2	4	4	2	3	19	63.33
3(0730-0830)	4	4	4	3	3	0	18	60.00
4(0930-1030)	2	1	3	5	3	2	16	53.33
5(1030-1130)	4	3	5	3	2	3	20	66.67
6(1130-1230)	1	4	3	3	3	2	16	53.33

[a]- samples were from five sources (hence n=5) being pallets, crates, truck bed, truck wheels, and drivers' boots.
[b]- n=4
[c]- n=29

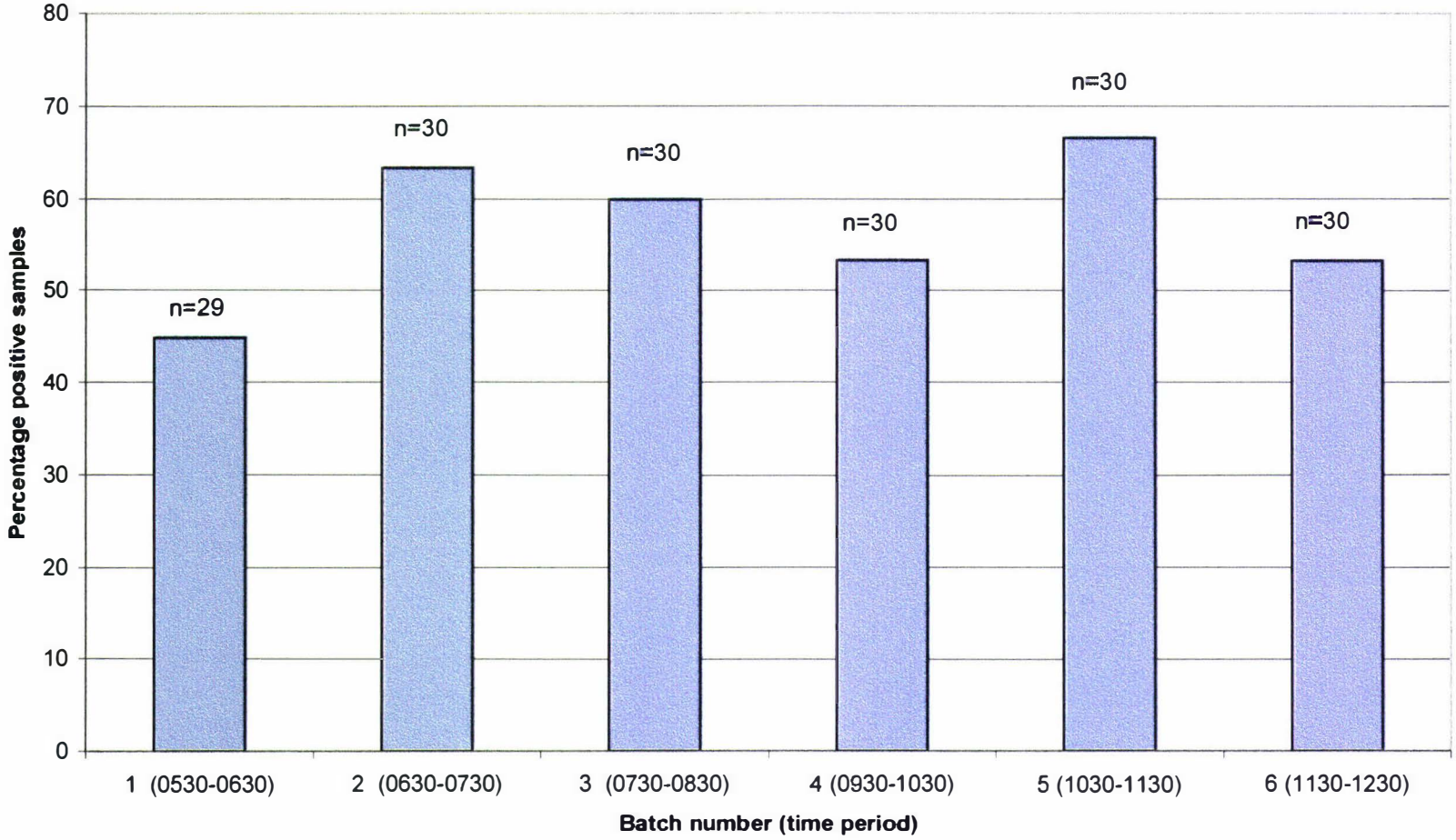


Figure 4.1 The level of *C. jejuni* contamination of slaughterhouse fomites at different time periods during the day

4.4 DISCUSSION

Studies to identify inanimate objects that are commonly contaminated with *C. jejuni* have previously been concentrated on the poultry farm and within the poultry processing plant (Shanker *et al.*, 1986; Evans, 1992; Jacobs-Reitsma *et al.*, 1995; Stern *et al.*, 1995). On the farm, it has been found that *C. jejuni* permeates the environment once the chickens become infected (Lindblom *et al.*, 1986; Gregory *et al.*, 1997). At the poultry processing plant, the organism has been isolated from a number of critical control points (Wempe *et al.*, 1983; Genigeorgis *et al.*, 1986). Previously, only a few attempts were made to isolate the organism from pallets, crates, trucks, tractors, forklifts, and the footwear of drivers and catchers all of which enter poultry farms during the catching of broilers for slaughter. In this study, all these fomites, except for tractor wheels, were found to be contaminated with *C. jejuni*. All the fomites were sampled after being washed just before they left for the farms, which suggests that they were still contaminated with viable *C. jejuni* when they reached the farms. They can therefore be regarded as potential sources of infection for broilers.

The only opportunity to sample tractor wheels was in the morning before the tractors left for the farms. They were returned and washed at the end of the day. The apparent absence of *C. jejuni* on tractor wheels could be due to a number of factors including effective cleaning with chlorinated water, death of *C. jejuni* from overnight exposure to high oxygen tension in the atmosphere and the transformation of the organism to a viable but non-culturable state (Rollins and Colwell, 1986). In terms of transmission of *C. jejuni* to broilers, the tractors would still contribute as they were reportedly moved between farms without being washed or disinfected.

It was observed during sampling that all the fomites had visible dirt and faecal materials on them. They were also wet from the washing except when they had dried overnight or over the weekend. *C. jejuni*, which is reported to be susceptible to drying, would presumably be protected by faecal material and the wet environment (Rollins and Colwell, 1986). Pallets were observed to have more dirt and faecal material than other equipment. However, there was no statistically significant difference between the prevalence of *C. jejuni* on pallets and that of other fomites.

With a mean *C. jejuni* isolation rate of 57.1%; pallets, crates, drivers' boots, catchers' boots and forklift wheels, should be considered important potential sources for transmission of the organism to broilers. This study confirms the findings of Giessen *et al.*, (1998) who isolated *C. jejuni* from slaughterhouse crates, trucks and truck tires. Their findings were however based on much fewer samples.

The epidemiological significance of the presence of *C. jejuni* on the footwear of slaughterhouse personnel, transport vehicles and other equipment is that they may introduce infection into hitherto *Campylobacter* free flocks during the first catch for slaughter. Contaminated lorries and crates may also transmit *C. jejuni* to broilers during transport to the abattoir (Stern *et al.*, 1995). In a practice where flocks are slaughtered in two or more batches rather than one, *C. jejuni* may spread amongst the birds remaining in the shed or even to other sheds in the farm, if it is introduced by the potential sources identified in this study. Although epidemiological evidence of such a transmission has not been reported yet, findings by other workers indicate that slaughtering flocks in more than one batch increases the prevalence of campylobacters (Berndston *et al.*, 1996; Hald *et al.*, 2000). Hald *et al.*, (2000) found that 50% of flocks that were slaughtered in a single batch were positive for thermophilic campylobacters. They also found that the flock prevalence rose to 85%, if flocks were exposed to abattoir equipment and personnel a week earlier when they thin the flocks by catching the first batch for slaughter. In another study, Wedderkopp *et al.*, (2000) found a single batch flock prevalence of 41% and a prevalence of 100% for flocks slaughtered in six batches. Dividing the flock into batches for staggered slaughter was thereafter considered a risk factor in the transmission of campylobacters to broilers.

In recognition of the risk of transmission of foodborne pathogens to broiler farms and contamination of chicken carcasses, transport vehicles, pallets, crates, and footwear of slaughterhouse personnel were routinely washed with water containing a high content of free chlorine (71.71 parts per million was obtained in a one off measurement of washing-bay water). Based on six consecutive batches of samples per day (five items sampled per batch) from 0530 hours to 1230 hrs and with the sampling repeated over six days (Table 4.3), it was found that there was no statistically significant difference

in the level of *C. jejuni* contamination at different times during the day. It appears that the washing serves to remove most of the visible dirt while the level of contamination with viable *C. jejuni* is maintained at the level reflected in Figure 4.1.

The results of this study identify specifically trucks, forklifts, pallets, crates, drivers' and catchers' boots as potential sources of *C. jejuni* for transmission to broilers. They also show that most of these fomites are still contaminated at the time they depart the slaughterhouse for farms to depopulate broiler flocks. The level of contamination of these fomites appears to remain the same during the day. Based on the findings of this study, it is recommended that all broiler flocks should be slaughtered in single rather than divided batches. That way the introduction of *C. jejuni* to broilers from slaughterhouse fomites could be avoided. It is also recommended that more effective cleaning and disinfection procedures be adopted at slaughterhouses to reduce the level of *C. jejuni* contamination of equipment and the footwear of personnel going to the poultry farms.

CHAPTER 5

5. EXPERIMENTAL TRANSMISSION OF *C. JEJUNI* IN BROILER CHICKENS

5.1 INTRODUCTION

Campylobacter enteritis is the commonest reported foodborne disease of humans in the developed countries and about half of the cases have been associated with the consumption of poultry meat (Lindblom *et al.*, 1986; Deming *et al.*, 1987; Skirrow, 1991; Evans, 1992). It is therefore widely accepted that the production of *Campylobacter* free broilers should significantly reduce human infections with *C. jejuni* (Evans, 1992; Giessen *et al.*, 1998).

Studies of the epidemiology of *C. jejuni* in broilers have identified several potential sources of the organism at the farm (Blaser *et al.*, 1983; Gregory *et al.*, 1997) and control measures targeting known risk factors are reported to reduce *C. jejuni* prevalence in broiler flocks (Giessen *et al.*, 1992; Kazwala *et al.*, 1992; Giessen *et al.*, 1996, 1998). Previous studies have suggested that the spread of *C. jejuni* in broilers is rapid (Smitherman *et al.*, 1984; Clark and Bueschkens, 1988; Stern and Meinersmann, 1989). But there is inadequate information about the rate of transmission of the organism once it is introduced to a susceptible flock. Therefore the main objective of this study was to determine the transmission rate of *C. jejuni* in broilers.

Boots have previously been implicated, but not confirmed, as a vector for the horizontal transmission of C. *jejuni* between broiler sheds in the farm (Lindblom *et al.*, 1986; Annan–Prah and Janc, 1988; Giessen *et al.*, 1998). An investigation was carried out concurrently with the transmission study to ascertain whether or not boots are capable of transmitting *C. jejuni* to susceptible broilers.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

Broiler chicks of the Ross 1 breed were purchased as day olds from a commercial hatchery. The chicks were of mixed sex. They were raised in a single pen at a stocking rate of 22 birds per m^2 until the age of 35 days. Temperature control, feeding regime and other production parameters were similar to those widely used in commercial poultry farms in New Zealand. At 35 days of age, the birds were allocated randomly into three groups. Group I had 10 birds. Their pen was in a separate shed to serve as a control. Group II comprising 100 birds remained in the original pen and formed the treatment group. Group III consisted of 10 birds, which were kept in a small pen 2.5 m away from the group II pen. They were used to assess the potential for transmission of the test strain of *C. jejuni* via boots. Figure 5.0 illustrates the set up of the pens.

All the birds were sampled by cloacal swabbing and tested negative for *Campylobacter* species at the age of seven days. Before the experiment started when the birds were 35 days old, they were all sampled prior to the introduction of an infected bird. All the 100 group II birds were sampled every 12 hours from the start to the end of the experiment. All the birds in the three groups were sampled at the end of the experiment. This last sample was 108 hours from the start of the experiment.

5.2.2 Biosecurity

A number of biosecurity measures were implemented during the experiment to prevent transmission of campylobacters from the environment, personnel and other possible sources of the bacteria. The shed used for raising the chicken was isolated from other poultry sheds and had not been used for over a year. The shed, which was considered inaccessible to rodents and insects, was cleaned and fumigated seven days before the introduction of the birds using a compound containing glutaraldehyde and ammonium chloride (Glutasan-QCT®; CHI-TEC detergents Ltd, Hamilton, New Zealand), prepared according to the manufacturer's instructions. A footbath with the same detergent was placed at the entrance to the shed to disinfect boots. The

disinfectant in the footbath was replaced every two days. Boots were changed when entering the shed so that only those kept within the shed were used when tending to the birds. Work clothes were also kept and used within the shed only. Disposable hand gloves were worn when tending to the birds and changed between experimental groups. Hands were washed with soap before and after tending to the birds.

5.2.3 Infection of the 'seeder' bird

The strain of *C. jejuni* used in the experiment had been isolated from a cloacal swab sample of a broiler chicken and identified by the method described in chapter 3. By the time it was used to inoculate the 'seeder' bird, it had been passaged about four to five times.

(i) Preparation of the inoculum

A Tryptic Soy Agar (TSA) plate was inoculated and streaked in the standard procedure with the experimental strain (*C. jejuni* 45) previously stored at –70°C in the culture collection. The plate was incubated at 42°C for 48 hours in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Single colonies from the TSA plate were inoculated onto five TSA plates that were incubated in the same conditions for 48 hrs. The growth from the plates was transferred with a sterile swab into a 30 ml universal container with 10 ml of Bolton's broth. The suspension was mixed with a vortex machine. A method for the enumeration of campylobacters described by Reid (1991) was used to determine the number of colony forming units (cfu) per ml of the suspension. Ten-fold dilutions of the suspension were made with 1% peptone water. Two TSA plates were plated with 0.1 ml each from each dilution. The plates were incubated for 48 hours under microaerophilic conditions. Four drops of pure glycerol on filter paper were placed at the bottom of the jar to absorb excess moisture and prevent spreading growth. The number of colonies was counted from both plates for each dilution and a formula described by Reid (1991), was used to calculate the number of cfu/ml in the original suspension. The bacterial suspension was stored at 4°C for 72 hours before it was used to infect the 'seeder' bird. It was reported by Kakoyiannis (1984) that *Campylobacter jejuni* could survive for at least 45 days at 4°C in a broth supplemented with ferrous sulphate, sodium pyruvate and sodium

metabisulfite (FBP broth). Bolton's broth contains super-oxide scavengers as did FBP broth and therefore it was assumed that it would maintain *C. jejuni*. The bacterial suspension remaining after the inoculation of the bird was immediately enumerated by the same method. The result obtained was used to calculate the number of cfu in the inoculum

(ii) Inoculation and monitoring

A chick was isolated at 29 days of age and inoculated orally with 7.78 x 10^6 cfu of *C. jejuni* 45. One ml of the inoculum was administered into the crop using a firm 1 mm bore-sized, 9 cm long plastic tube attached to a 5 ml plastic syringe by a blunt metal needle (Plate 5.1a and 5.1b). Faecal samples and cloacal swab samples were taken daily starting from 24 hours after inoculation and cultured for *C. jejuni*. At 32 days of age a faecal sample from the chick was enumerated for *C. jejuni* using the method described by Reid (1991). The chick was found to be excreting 2.45 x 10^5 cfu per gram of faeces. Both faecal and cloacal swab samples from the chick were positive for *C. jejuni* from 24 hrs after inoculation to the morning when the chick was introduced to the birds in group II.

5.2.4 The boot study

Once in the morning and the evening, the boots worn while tending to group II birds, were used in group III pen. The operator simulated on-farm practice by entering the pen and walking in it checking drinkers and feeders. The simulation exercise lasted for one to two minutes each time for the four and half days of the experiment.

5.2.5 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis of the isolates was performed using a modification of a method described by On and Vandamme (1997). Sixteen isolates from the 100 chickens in the treatment group, and four from the boot study group, were selected randomly for PFGE to confirm them as the experimental strain. Pure 48-hour cultures obtained buy sub-culturing single colonies from each of the isolates, were harvested using sterile swabs, into 3 ml aliquots of brain heart infusion (BHI) broth. The optical

density (OD) of each of the bacterial suspensions in BHI broth was measured with a Helios Alpha® (Unicam, Cambridge, UK) spectrophotometer. Two hundred microliters of cell suspension with an OD of 1.4 when measured at 610 nm, was drawn from each isolate and placed in an eppendorf tube and then centrifuged at 13000 revolutions per minute (rpm) for five minutes. After removing the supernatant, the cells were re-suspended in 150 µl of cold PETT IV buffer (1M NaCl, 10 mM Tris-HCl (BDH laboratory supplies, Poole, England) [pH 8.0], 10 mM ethylenediamine tetra-acetic acid (EDTA) (BDH laboratory supplies, Poole, England) [pH 8.0]) and centrifuged for five minutes at 13000 rpm. The supernatant was removed and the pellet was re-suspended in 50 µl of PETT IV buffer.

A 100 µl of molten agarose (4 ml PETT IV buffer, 40 mg low melt agarose (Bio-Rad Laboratories, California, USA) heated in a boiling water bath until the agarose dissolved and then cooled) was added to each cell suspension and pipetted up and down to mix and immediately placed into plug moulds. The plug moulds were cooled on ice for an hour and then each of them was placed into 1 ml of lysis buffer (0.5 M EDTA, 1% sodium lauroyl sarcosine (BDH laboratory supplies, Poole, England), 0.1% proteinase K (Roch Diagnostics, Manheim, Germany)) in eppendorf tubes. The tubes were incubated at 56°C for 24 hours.

After incubation, the plugs were transferred into plastic universal bottles each containing 10 ml TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). They were then placed on ice and incubated for an hour on a rocking machine. The buffer was drained off and replaced with another 10 ml TE buffer. The universals were re-incubated in the same manner for another hour. The washing step was repeated three more times. Each of the plugs was stored in an eppendorf tube containing 1 ml of sterile TE buffer and kept at 4°C until required.

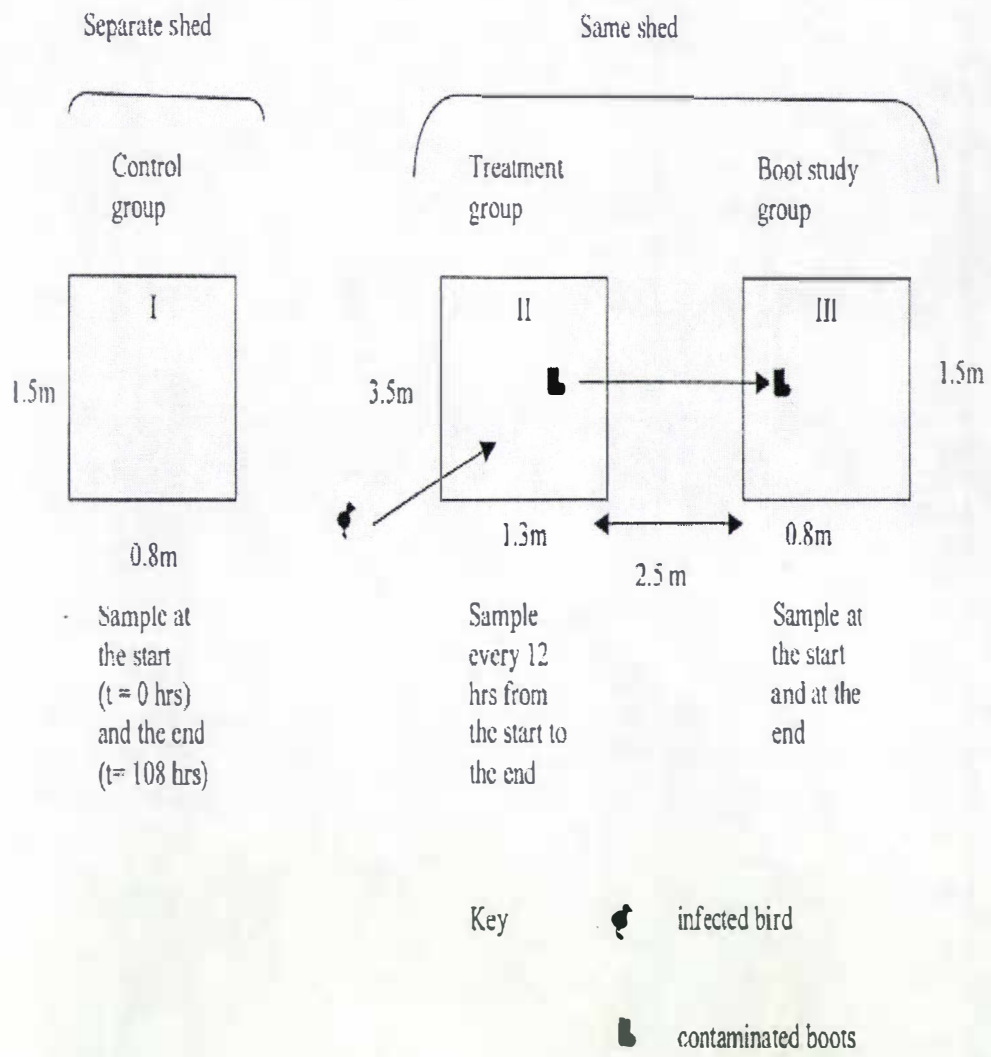
One-third portion of each plug was placed into 100 µl of restriction buffer (*Sma*I NEB [12 µl of 10X NE buffer 4 (New England Biolabs, MA, USA), 88 µl sterile milli Q® water (Milli-pore, Intertech, Bedford, MA, USA)]) and equilibrated on ice for 45 minutes. The restriction buffer was then removed and replaced with 100 µl of cutting buffer (10 µl of 10X NE buffer 4, 1.5 µl of 20 Units/µl *Sma*I (New England, Biolabs, MA, USA), 88.5 µl sterile Milli-Q water) and the plugs were kept on ice for 45

minutes to equilibrate. The plugs were then incubated in the cutting buffer at 25°C for 24 hours.

A wide and long 1% pulsed-field certified agarose was prepared by adding 140 ml of a ten-fold dilution of 5X TBE buffer (0.45 M Tris-HCl, 0.45 M boric acid (BDH laboratory supplies, Poole, England), 10 mM EDTA) to 1.4 g pulsed-field certified agarose (Bio-Rad laboratories, California, USA). The mixture was heated in a microwave oven for three minutes until the agarose dissolved and was cooled in a water bath to 50°C. The agarose was poured into a gel mould with a comb and allowed to solidify for an hour before the comb was removed. It was placed in a gel tank containing 2.5 L of 0.5X TBE buffer, which had been circulated through the tank for an hour at 14°C. The gel was pre-electrophoresed at 6 V/cm, pulse times of 5 seconds, for an hour using CHEF MAPPER® apparatus (Bio-Rad laboratories CA, USA).

The gel was removed from the chamber and the buffer was removed from the wells with a pipette. The plugs were loaded into the wells and pushed to the bottom to remove air bubbles. A low-molecular-weight marker (New England Biolabs, MA, USA) and a λ-marker (New England Biolabs, MA, USA) were also loaded into the wells. The gel was put back in the chamber and run at 6 V/cm for 22.5 hours with an initial switch time of 0.5 seconds and a final switch time of 40 seconds. The temperature of the buffer was set at 14°C. The gel was stained in a solution containing 1 µg/ml ethidium bromide for ten minutes, and rinsed in milli- Q water. It was trans-illuminated under ultra-violet light, and photographs were taken.

Figure 5.0 An illustration of the experimental set up



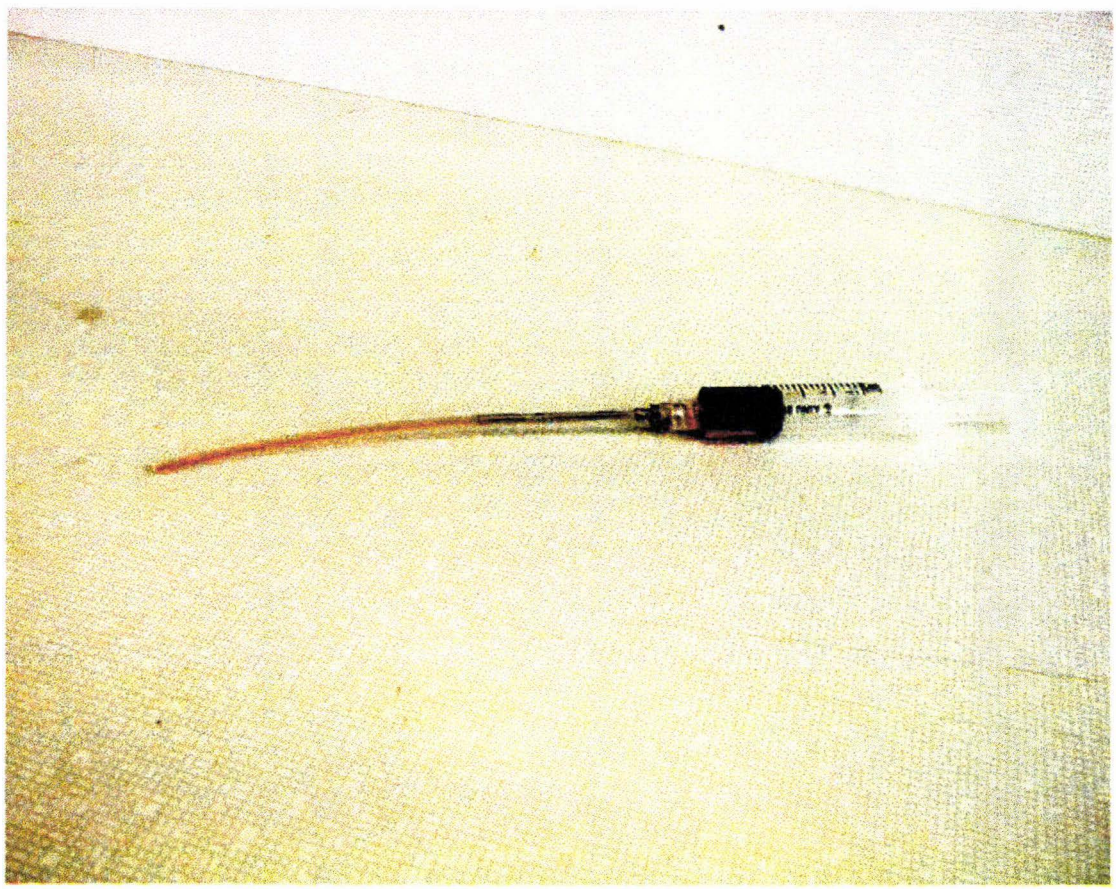


Plate 5.1a The syringe used to inoculate the 'seeder' bird showing the attached plastic tube.

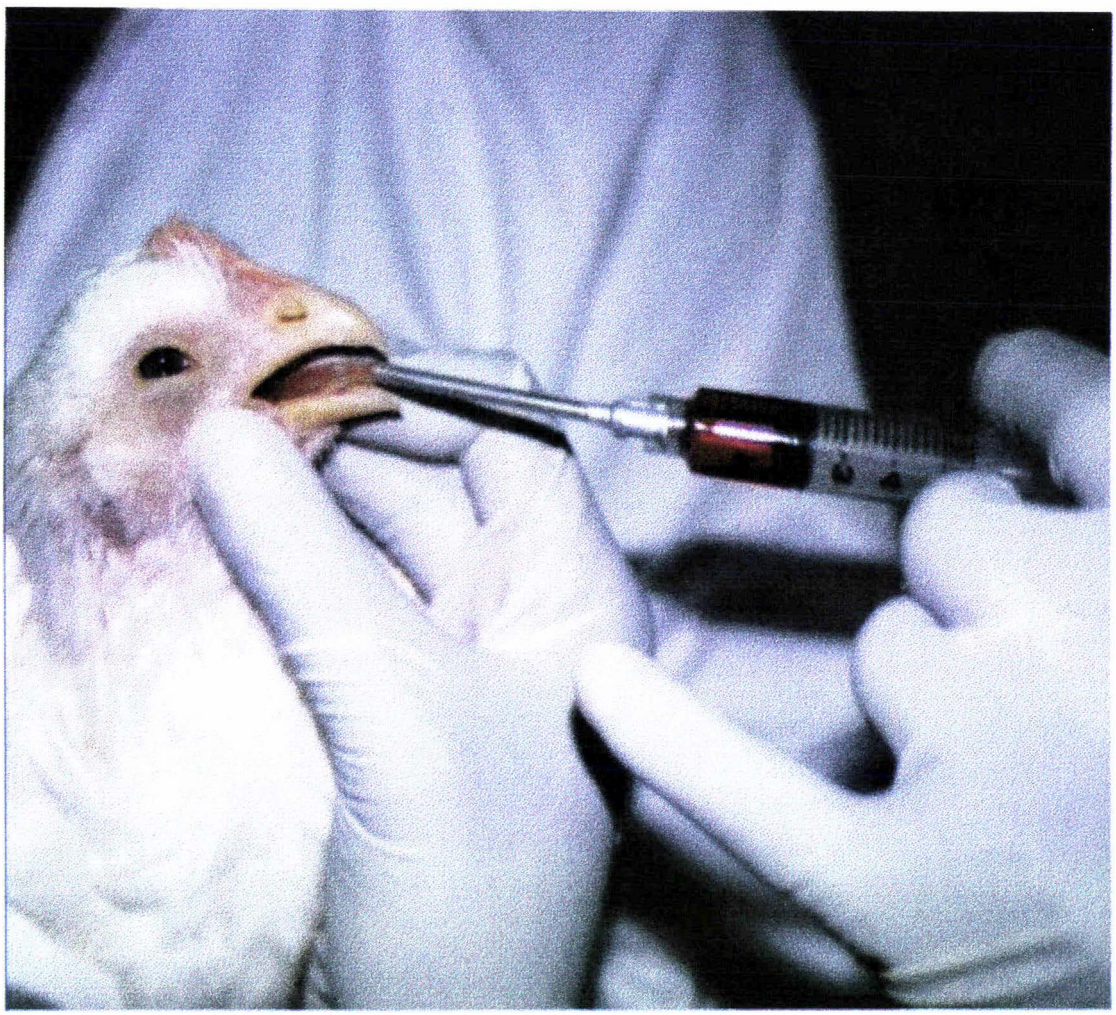


Plate 5.1b The inoculation of the 'seeder' bird with the experimental strain of *Campylobacter jejuni*

5.3 RESULTS

Campylobacter jejuni was naturally transmitted from an experimentally infected bird to susceptible birds within 12 hours of the bird being introduced to the *Campylobacter* free birds (Table 5.1). The infection spread rapidly resulting in 100% infection by 48 hours from the start of the experiment, and all the birds remained infected until the end of the experiment (Figure 5.1). All the ten birds in the control group were still not infected at the end of the experiment. On the other hand, all the ten birds in the boot study group were positive for *C. jejuni* at the end of the experiment (Table 5.1).

The number of new infections in the treatment group rose rapidly and peaked at 24 hours from the time the infected bird was introduced (Table 5.2). A graphical representation of the transmission rate of *C. jejuni* in the treatment group resembled an epidemic curve (Figure 5.2).

An infection parameter, beta (β) was calculated using a formula for a simple deterministic epidemic in continuous time (Daley and Gani, 1999) and the value of β was $2.1 \times 10^{-3} \pm 0.013 \times 10^{-3}$ (mean $\pm$ standard error). Using the simple deterministic epidemic model from Daley and Gani (1999) and $\beta = 0.002$, data predicting the course of *C. jejuni* transmission in a population of 100 birds was generated (Table 5.3; Figure 5.3a, and Figure 5.3b). According to the model, the transmission rate would peak 26 hours from the time the infected bird is introduced and all the birds would be infected by 50 hours. The transmission rate in a 100 birds, predicted by the model (Table 5.3 and Figure 5.3b), was found to be remarkably similar to that obtained in the experiment (Table 5.2 and Figure 5.2). However, when the population size was increased to 10,000 or 20,000 the period to completion of the epidemic, predicted by the model, was found to be less than four hours, which is unrealistic.

Isolates from the treatment group were shown to be similar to the experimental strain of *C. jejuni* ($p<0.05$) using pulsed-field gel elctrophoresis of genomic DNA (Figure 5.4). Four isolates from the boot study group were also similar to the experimental strain.

Table 5.1 Isolation of *C. jejuni* from a group of broilers exposed to a 'seeder' bird and another group exposed to contaminated boots

Time (hours)	Number of positive birds		
	Control group (n = 10)	Treatment group (n = 100)	Boot study group (n = 10)
0	0	1*	0
12	ND	12	ND
24	ND	67	ND
36	ND	96	ND
48	ND	100	ND
60	ND	100	ND
72	ND	100	ND
84	ND	100	ND
96	ND	100	ND
108	0	100	10

* - The positive bird at time = 0 hrs, was the 'seeder' bird.
ND – not done

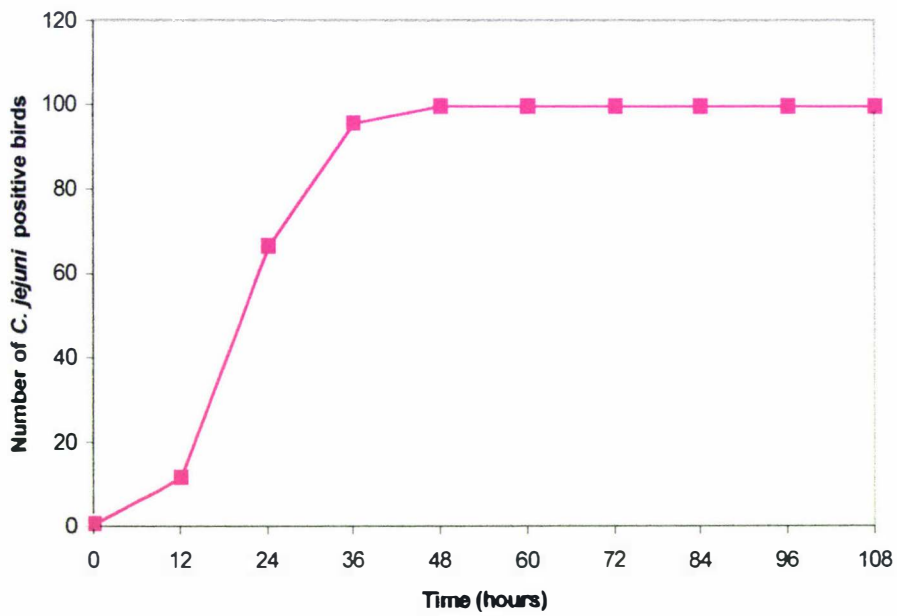


Figure 5.1 The spread of *C. jejuni* with time after the introduction of an infected bird

Table 5.2 The number of new *C. jejuni* positive birds against time, after the introduction of the infected bird.

Time (hours)	No. of new positive birds (n=100)
0	1*
12	11
24	55
36	29
48	4
60	0
72	0
84	0
96	0
108	0

*-The positive bird at time =0hours, was the 'seeder' bird.

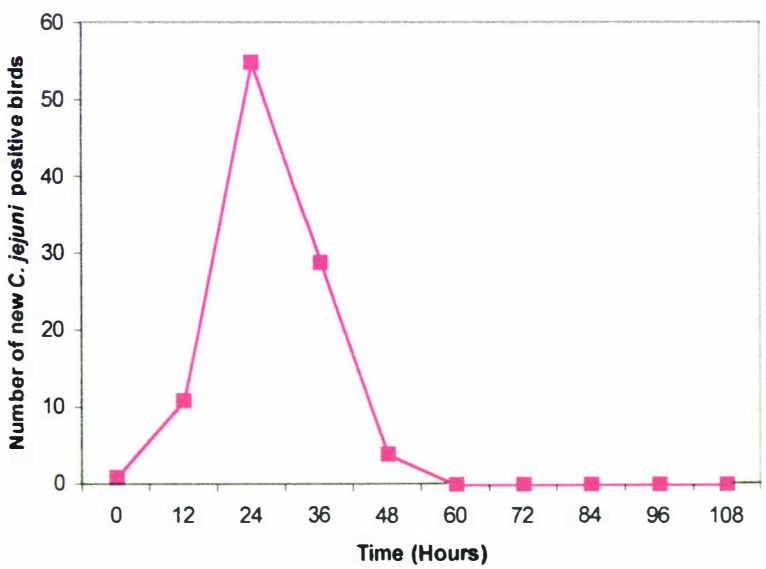


Figure 5.2 The transmission rate of *C. jejuni* in a group of susceptible birds.

Table 5.3 The number of new infections per hour and the total number of infections over time, in a population of 100 birds, based on a simple deterministic epidemic model.[a]

Time (hours)	New infections	Total no.infected	Time (hours)	New infections	Total no.infected
1	0.2	1.2	37	1.52	93.25
2	0.24	1.43	38	1.26	94.51
3	0.28	1.72	39	1.04	95.54
4	0.34	2.06	40	0.85	96.4
5	0.4	2.46	41	0.69	97.09
6	0.48	2.94	42	0.57	97.66
7	0.57	3.51	43	0.46	98.11
8	0.68	4.18	44	0.37	98.48
9	0.8	4.99	45	0.3	98.78
10	0.95	5.93	46	0.24	99.02
11	1.12	7.05	47	0.19	99.22
12	1.31	8.36	48	0.16	99.37
13	1.53	9.89	49	0.12	99.5
14	1.78	11.68	50	0.1	99.6
15	2.06	13.74	51	0.08	99.68
16	2.37	16.11	52	0.06	99.74
17	2.7	18.81	53	0.05	99.79
18	3.05	21.87	54	0.04	99.83
19	3.42	25.28	55	0.03	99.87
20	3.78	29.06	56	0.02	99.89
21	4.12	33.18	57	0.02	99.92
22	4.43	37.62	58	0.01	99.93
23	4.69	42.31	59	0.01	99.95
24	4.88	47.19	60	0.01	99.96
25	4.98	52.18	61	0.01	99.97
26	4.99	57.17	62	0.01	99.97
27	4.9	62.07	63	0	99.98
28	4.71	66.77	64	0	99.98
29	4.44	71.21	65	0	99.99
30	4.1	75.31	66	0	99.99
31	3.72	79.03	67	0	99.99
32	3.31	82.34	68	0	99.99
33	2.91	85.25	69	0	99.99
34	2.51	87.77	70	0	100
35	2.15	89.91	71	0	100
36	1.81	91.73	72	0	100

[a] The number infected at t=0hrs is 1, and beta (β) = 0.002 as it was calculated from the results of the experiment using the following equation;

$$\beta Nt = \ln\frac{y(t)}{N - y(t)} - \ln\frac{y0}{N - y0}$$

where N denotes population size, *y(t)* and *y0* denote the number infected at time t, and t = 0 hrs respectively, and t is the time in hours (Daley and Gani, 1999).

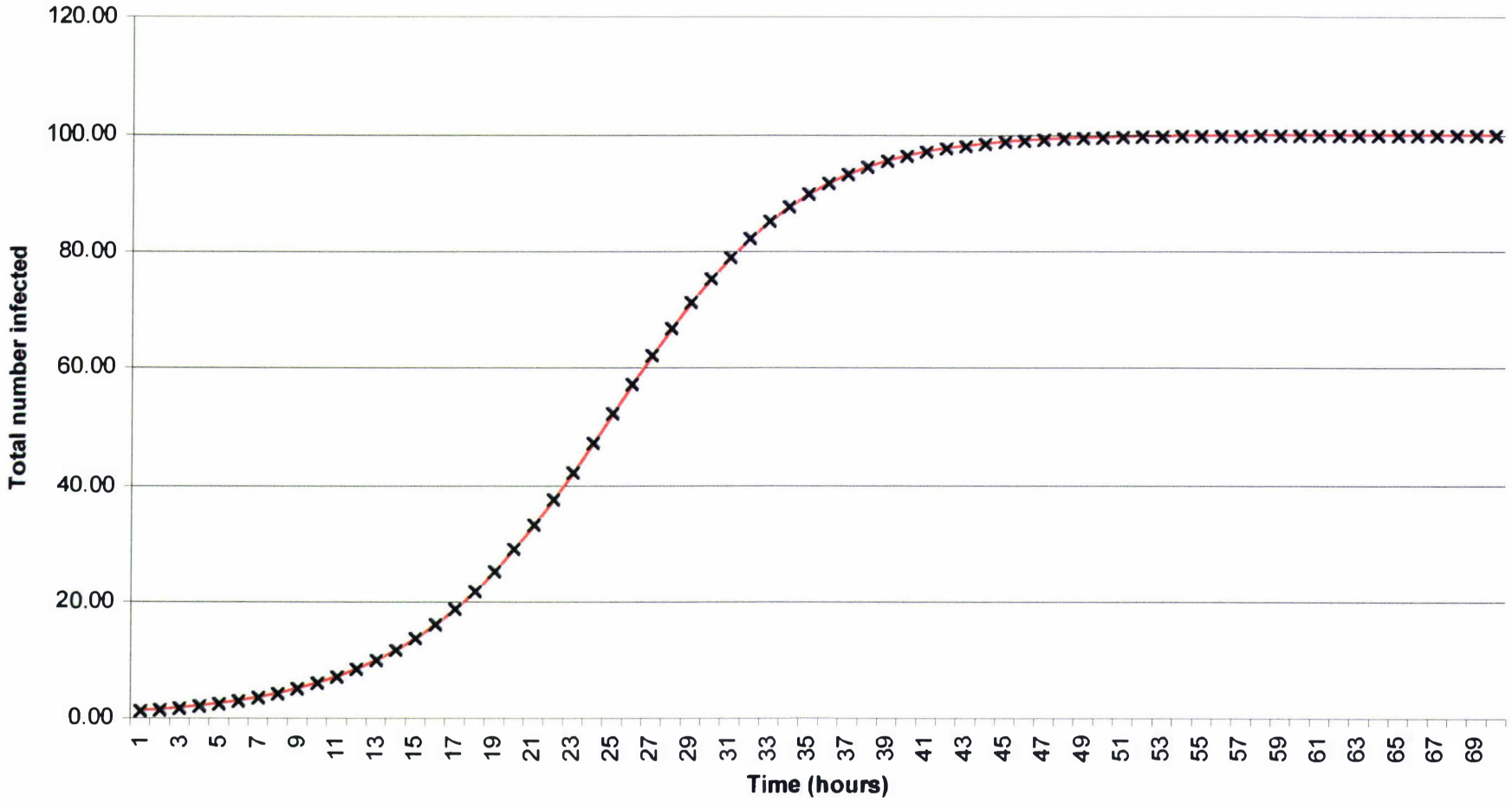


Figure 5.3a The spread of *C. jejuni* in a population of a 100 birds based on a simple deterministic epidemic model

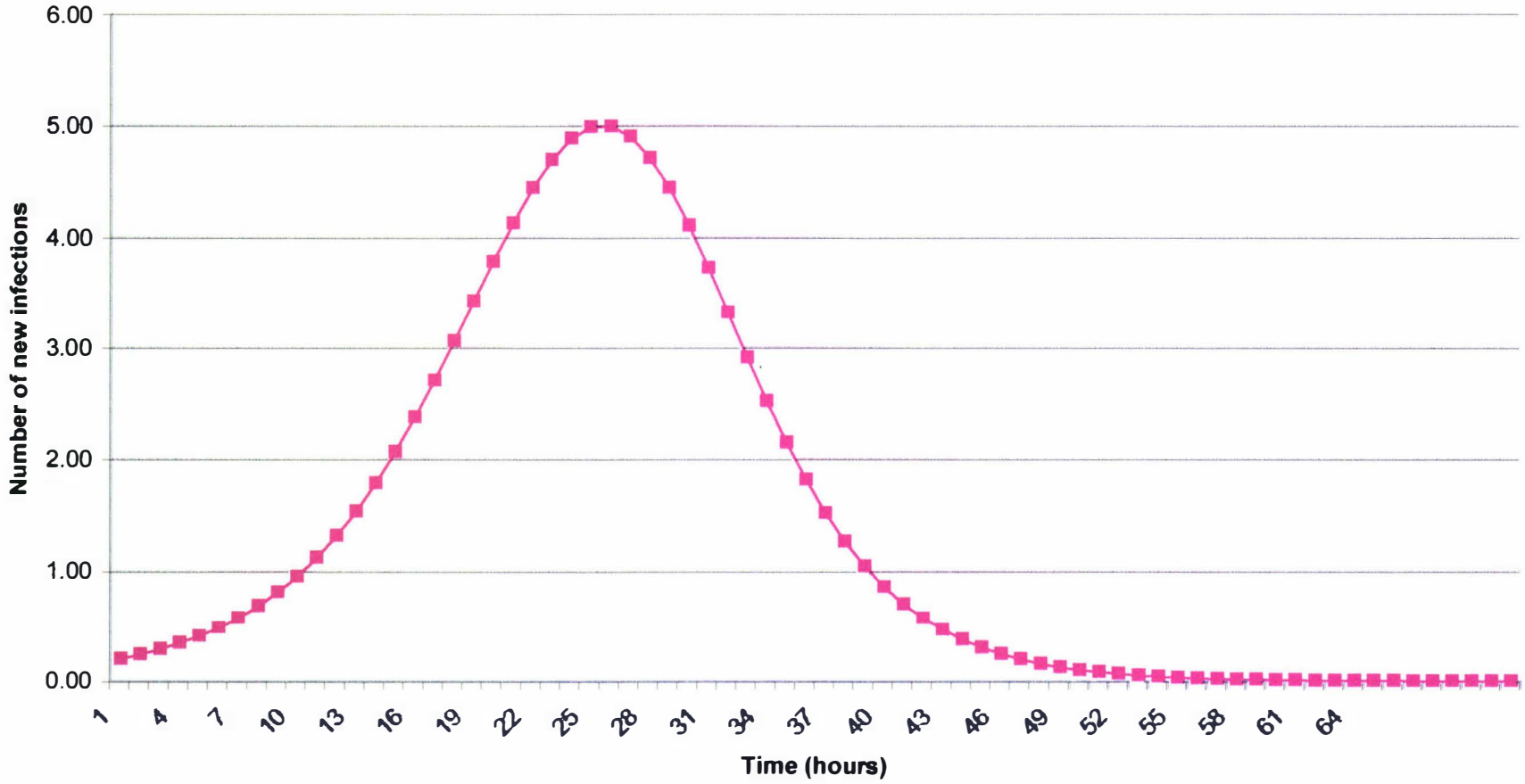


Figure 5.3b The transmission rate of *C. jejuni* in a 100 birds based on a simple deterministic epidemic model

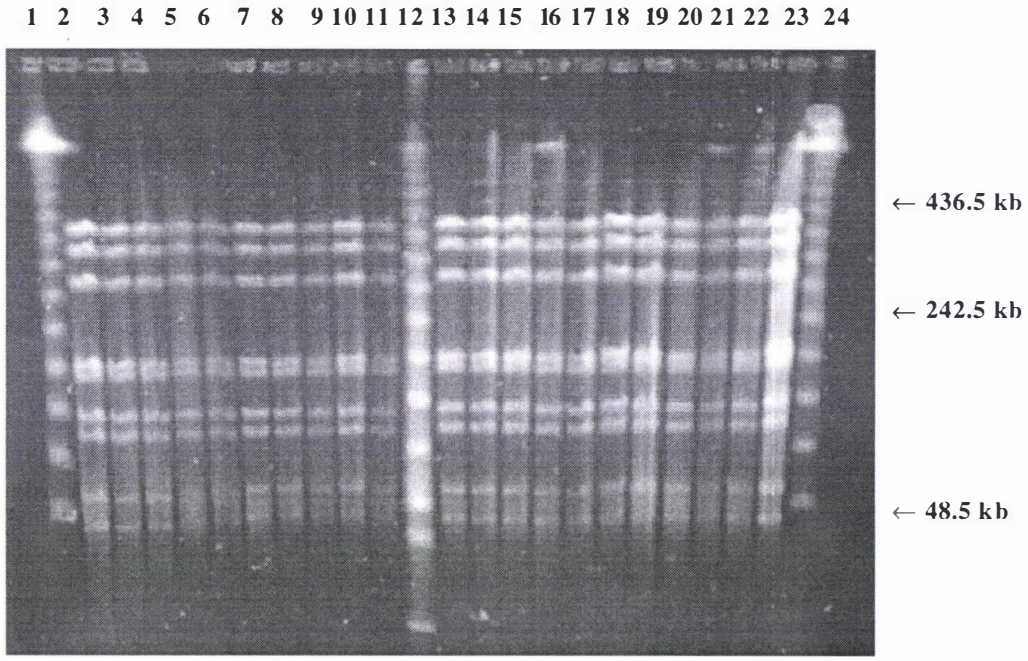


Figure 5.4 PFGE profiles of chromosomal DNA of *C. jejuni* isolates from the treatment and boot study groups, digested with restriction endonuclease *Sma*I. Lane 1 and 24 contain molecular weight marker (λ-ladder). Lane 12 contains low molecular weight marker. Lanes 2 to 11 and 13 to 18 contain isolates from the treatment group. Lanes 19 to 22 contain isolates from the boot study group. Lane 23 contains the isolate from –70°C before it was used in the experiment.

5.4 DISCUSION

This study demonstrated that *C. jejuni* could be naturally transmitted to the rest of susceptible broilers from one experimentally infected bird, in an environment similar to commercial poultry farming conditions. Previous experiments demonstrated the same form of transmission but did not simulate commercial farming conditions (Clark and Bueschkens, 1988; Shanker *et al.*, 1990; Kazwala *et al.*, 1992; Kakoyiannis, 1984). It took less than 12 hours for susceptible birds to be colonised and excrete *C. jejuni,* which suggests that the organism is highly infectious for broilers. In previous studies, *C. jejuni* was isolated from susceptible birds 24 hours after exposure to an infected bird (Kakoyiannis, 1984; Clark and Bueshkens, 1988).

C. jejuni has been reported to spread from a few infected birds to most susceptible birds in less than 72 hours and to the rest of the birds within a week even in flocks of 20, 000 birds (Smitherman *et al.*, 1984; Montrose *et al.*, 1985; Clark and Bueshkens, 1988; Shanker *et al.*, 1990). In this study, the infection took a slightly less time of 36 hours to spread to 96% of the birds and 48 hours to spread to the rest of the birds. In this study, all the birds were handled every 12 hours during sampling, which resulted in extra mixing of the birds. These manipulations could have enhanced the spread of the infection.

Birds that became infected at 35 days of age, remained infected until 40 days of age confirming the finding by Jacobs-Reitsma *et al.*, (1995) that once infected, broilers remain colonised by *C. jejuni* until slaughter. The birds did not show any signs of ill health after infection. Therefore unless epidemiological surveillance is done in which faecal or cloacal swab samples are taken from flocks and tested for *C. jejuni*, it may not be possible to know whether or not a flock is infected.

The results of *C. jejuni* transmission from this study were able to fit into a simple deterministic epidemic model suggesting, as it is assumed by the model, that *C. jejuni* is highly infectious to broilers with a very short pre-patent period, the infection is non-pathogenic, and the birds were mixing homogeneously. Cawthraw *et al.*, (1996) reported that as few as 40 *C. jejuni* bacterial cells are required to infect broiler chicks. Montrose *et al.*, (1985) found that *C. jejuni* colonises the palatine mucosa of broilers, which would enhance transmission through drinkers. Both these findings support the rapid spread of *C. jejuni* observed in this study. The pattern of transmission of *C. jejuni* in broilers observed in this study appears comparable to the transmission of some contagious human diseases such as influenza and measles.

According to Anderson and May (1992) the transmission coefficient β, determines the rate at which new infections arise as a consequence of interaction between susceptible and infective individuals. Beta (β) had also previously been defined by Frauenthal (1980) as "the average number of contacts between susceptible and infected individuals which lead to a new infective per unit of time per infective per susceptible in the population". Therefore β characterises the infection and as further explained by Anderson and May (1992) β reflects epidemiological, environmental and biological

factors that affect the transmission rate. However, β cannot be measured directly. As it was not possible to predict the transmission rate of *C. jejuni* in larger broiler populations using β from this study, it is speculated that β would be different in such populations and can only be determined by carrying out experiments similar to this one but with larger population sizes. In larger populations, homogeneous mixing is less likely and therefore the transmission rate may be lower than that observed in this study.

The recovery of *C. jejuni* from chickens that were exposed to contaminated boots confirms that boots can mechanically transmit the organism. Previous epidemiological studies had led to the speculation that footwear caused horizontal transmission of *C. jejuni* in broiler farms (Annan-Prah and Janc 1988; Gregory *et al.*, 1997; Giessen *et al.*, 1998). As a result disinfection of boots at the entrance to broiler sheds or changing boots between sheds has been widely adopted as a biosecurity measure to prevent the introduction of *C. jejuni* to broilers.

This study has confirmed speculations that *C. jejuni* spreads rapidly in broiler flocks under commercial farming conditions. It has also demonstrated that contaminated boots can act as a vehicle for horizontal transmission of *C. jejuni*. Unlike previous studies, the experiment has permitted the calculation of a transmission parameter, β, which is thought to vary with factors that influence transmission rates particularly population sizes. It is recommended that studies similar to this one should be carried out with larger population sizes, as transmission rates obtained would represent those in commercial farms where broiler populations are large.

CHAPTER 6

6. GENERAL DISCUSSION

It is widely believed that broiler flocks are colonised by *C. jejuni* from unknown sources outside the poultry house (Kazwala *et al.*, 1990; Hald *et al.*, 2000). The presence of the organism in both animate and inanimate environmental reservoirs is well documented (Annan-Prah and Janc, 1988; Stern, 1992; Nesbit *et al.*, 2001). In contrast, the route of transmission from the known reservoirs to broilers remains unclear. A possible route of transmission identified by epidemiological studies, is the entry into broiler houses, of slaughterhouse personnel and the fomites used during partial depopulation of broiler flocks (Jacobs-Reitsma *et al.*, 1995; Hald *et al.*, 2000; Wedderkopp *et al.*, 2000). This study identified the following slaughterhouse fomites as potential sources of *C. jejuni* for broilers: drivers' boots, catchers' boots, truck bed, truck wheels, forklift wheels, pallets, and crates. It was found that 111 (53.11%) of 209 samples taken from these fomites were positive for *C. jejuni.* Although Giessen *et al.*, (1998) only took a few swab samples from slaughterhouse crates and a lorry during partial depopulation, they found them to be contaminated with the organism, which is in agreement with the findings in this study.

Despite the lack of comparative data, the prevalence of *C. jejuni* in pallets (75%), crates (58.33%), truck bed (47.22%), truck wheels (50%), forklift wheels (31.25%), drivers' boots (54.29%), and catchers' boots (66.67%) can be considered significantly high. As samples were taken from all fomites just before they left the slaughterhouse for broiler farms, it can be assumed that the level of *C. jejuni* contamination on them would still be high when they reach the farms. In addition, *C. jejuni* is known to survive for up to three weeks in faecal material and a wet environment (Blaser *et al.*, 1980b) both of which were observed on the fomites. Further investigation by epidemiological studies and molecular typing techniques is required to definitively determine whether *C. jejuni* on slaughterhouse fomites causes infections in broiler flocks particularly in the period following the entry of these fomites into broiler houses or farms.

Stern and Meinersmann (1989), Stern *et al.*, (1995), and Giessen *et al.*, (1998) suggested that *C. jejuni* could be transmitted from contaminated crates and lorries to *campylobacter* free broiler chickens during transport to the slaughterhouse. In this study, over 58% and 47% of samples from crates and truck bed respectively, were positive for *C. jejuni.* Conceivably, these fomites could serve as a source of *C. jejuni* infection for broilers during transport. The fomites may also cause external contamination of broiler chickens with *C. jejuni* during transport (Stern *et al.*, 1995).

During this study, trucks, forklifts, tractors, pallets and crates were washed with pressurized chlorinated water to control bacterial contamination on them. This cleaning procedure did not seem effective as evidenced by a consistently high level of contamination on fomites throughout each day during the six days over which the study was performed (Table 4.3 and Figure 4.1). Wang *et al.*, (1983) found that a free chlorine concentration of 5 parts per million (ppm) in a disinfectant, was sufficient to kill up to 10^4 *C. jejuni* cells within 1 minute. It is also documented that free chlorine concentrations of up to 50 ppm in water, are effective in killing free or unattached microorganisms (Bryan and Doyle, 1995). But, free chlorine, even in high concentrations of up to 50 ppm, has little effect on *C. jejuni* and other bacteria if the organisms are attached to or entrapped in organic material (Wang *et al.*, 1983; Bryan and Doyle, 1995). Therefore, the presence of faecal material on slaughterhouse fomites may have prevented the killing of *C. jejuni* by the relatively high (up to 70 ppm) free chlorine concentration of the washing-bay water. The development and implementation of alternative practical methods of eliminating or significantly reducing *C. jejuni* found on slaughterhouse fomites should reduce the risk of transmission of the organism to broilers at the time of slaughter.

Boots have already been identified by bacteriological culture methods as potential vectors in horizontal transmission of *C. jejuni* to broiler flocks from sources external to the broiler house and between broiler flocks in separate sheds. (Lindblom *et al.*, 1986; Annan-Prah and Janc, 1988; Gregory *et al.*, 1997; Giessen *et al.*, 1998; Hald *et al.*, 2000). In support of this role of boots in *C. jejuni* transmission, Humphrey *et al.*, (1993) found that when workers dipped their boots in a disinfectant before entering the broiler house, the colonization of broiler flocks with the organism was either

delayed or prevented. Also, Hald *et al.*, (2000) found the lack of disinfection of workers' boots at the entrance of the broiler house to be associated with an increased risk of occurrence of *C. jejuni* in broiler flocks. In this study, it was confirmed using pulsed-field gel electrophoresis that an experimental strain of *C. jejuni* could be mechanically transmitted from infected broiler chickens to those that are susceptible, by boots. Therefore the finding of this study supports existing biosecurity procedures that require the dipping of boots in a disinfectant at the entrance to the broiler house or a change to footwear dedicated for use within the broiler house (White *et al.*, 1997; Giessen *et al.*, 1998; Hald *et al.*, 2000).

By carrying out an experimental transmission study with a 'seeder' bird and 99 susceptible broilers at slaughter age, it was demonstrated quantitatively that *C. jejuni* spreads rapidly in broiler chickens (Figure 5.1 and 5.2). The study was designed to closely mimic natural infection therefore the birds were raised under conditions similar to those in commercial farms. With respect to a rapid transmission rate, the finding of this study is in agreement with those of previous investigators who monitored *C. jejuni* infection in commercial broiler flocks and found that virtually all the samples became positive within a week of obtaining the first positive sample (Smitherman *et al.*, 1984; Genigeorgis *et al.*, 1986; Shreeve *et al.*, 2000).

Moreover, the fact that susceptible birds were infected and excreted *C. jejuni* within 12 hours of exposure to the 'seeder' bird suggests that the pre-patent period of *C. jejuni* in broilers could be shorter than 12 hours. In that case, *Campylobacter* free birds could become infected and excrete the organism during transport and while they are held in crates before slaughter, if they are exposed to *C. jejuni* found on slaughterhouse trucks and crates. An investigation with sampling intervals less than 12 hours should be carried out to determine the accurate pre-patent period of *C. jejuni* in broiler chickens.

The fact that the data obtained from the experiment could fit into a simple deterministic epidemic model suggests that the transmission rate of *C. jejuni* in broilers may be amenable to study by mathematical models. Of interest is that when a coefficient β, calculated using the experimental data and a formula for a simple deterministic epidemic curve was used to predict the course of *C. jejuni* transmission

in a population of 100 birds, it was found that the predicted transmission rate was very similar to that obtained by experiment. Such similarity may indicate that the conditions in the broiler house were similar to those assumed in the epidemic model the main ones being that *C. jejuni* is highly infectious but non-pathogenic to broilers, *C. jejuni* infection in broilers has a very short pre-patent period, and the birds mix homogeneously (Frauenthal, 1980; Anderson and May, 1992).

Although it was not possible to predict the transmission rate of *C. jejuni* in large broiler populations using the simple deterministic epidemic model, other workers have reported that most birds in a flock quickly become colonised after the introduction of the infection (Shreeve *et al.*, 2000; Gibbens *et al.*, 2001). In this respect, it has been suggested that the rapid transmission is aided by the following: contamination of the internal environment of the broiler house including feed, litter, water, and air (Engvall *et al.*, 1986; Evans, 1992; Gregory *et al.*, 1997), an increase in the colonization potential of the bacterial strain acquired by *in vivo* passage (Sang *et al.*, 1989; Cawthraw *et al.*, 1996), a low infective dose that can be 35 to 40 bacterial cells (Stern *et al.*, 1988; Cawthraw *et al.*, 1996), and coprophagy. The transmission rate has also been found to vary depending on the challenge strain (Shanker *et al.*, 1990). On more than one occasion, investigators have alluded to the population size of the flock having little or no effect on the transmission rate of *C. jejuni* (Stern, 1992; Evans and Sayers, 2000; Gibbens *et al.*, 2001). Therefore the transmission rate obtained in this study could closely approximate that which would be obtained in large commercial broiler populations. Nevertheless, it is recommended that further studies of the transmission rate of *C. jejuni* in broilers be carried out using larger population sizes.

6.1 CONCLUSIONS

The following conclusions are drawn from the findings of the studies discussed above:

- The footwear of slaughterhouse personnel and slaughterhouse fomites namely trucks, forklifts, pallets, and crates leave the processing plant for farms highly contaminated with *C. jejuni* and therefore they are potential sources for transmission of the organism to broiler chickens.

- Cleaning slaughterhouse fomites with pressurized chlorinated water is ineffective in eliminating or significantly reducing *C. jejuni* found contaminating them.

- Boots can mechanically transmit *C. jejuni* from an infected group of broiler chickens to susceptible birds.

- The pre-patent period of *C. jejuni* in broiler chickens is less than 12 hours.

- The transmission rate of *C. jejuni* in broiler chickens is in the form of a simple epidemic. The epidemic is characterized by a transmission coefficient β that is not amenable to direct measurement but can be calculated using experimental data and a mathematical formula.

APPENDICES

Appendix 1. Preparation of Bolton's Broth

Steps

1. Weigh 27.6 grams powder of *Campylobacter* enrichment broth (Lab M; Lab 135), disperse in 1 litre of distilled water and allow soaking for 10 minutes. Swirl to mix.
2. Sterilize by autoclaving at 121° C for 15 minutes.
3. Cool to 47° C.
4. Reconstitute 2 vials of Lab M x131 by adding to each vial, 5 ml of 50% ethanol, using a sterile needle and syringe.
5. Add 2 vials of reconstituted Lab M x131 to 1 litre of *Campylobacter* enrichment broth after cooling to 47° C (see step 3 above).
6. Add 50 ml of saponin lysed horse blood.
7. Mix well before dispensing.

Lab M; Lab 135 (*Campylobacter* enrichment broth) [1]

Formula

	Grams per litre
Meat peptone	10.0
Lactalbumin hydrolysates	5.0
Yeast extract	5.0
Sodium chloride	5.0
Haemin	0.01
Sodium pyruvate	0.5
α - ketoglutaric acid	1.0
Sodium metabisulphite	0.5
Sodium carbonate	0.6

Final pH 7.4 ± 0.2

Lab M x131[1]

Vial contents

	per vial	mg per litre
Cefoperazone	10 mg	20
Vancomycin	10 mg	20
Trimethoprim	10 mg	20
Cycloheximide	25 mg	50

[1] – Topley House, Bury, England.

Appendix 2. Preparation of Blood Agar

The medium consists of a blood base on top of a salt base.

Steps for salt base

1. Suspend 15.0 grams of agar in 1 litre of distilled water.
2. Add 5.0 grams sodium chloride and heat to dissolve.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Cool to 50° C.
5. Pour 10 ml amounts into sterile petri dishes and allow to cool and dry.

Steps for blood base

1. Suspend 42.0 grams of Columbia agar powder in 1 litre of distilled water and heat to boiling to dissolve completely.
2. Sterilize by autoclaving at 121°C for 15 minutes.
3. Cool to 50° C.
4. Add 5% (v/v) of sheep blood and mix.
5. Pour 10 – 15 ml amounts over an already prepared salt base (see above).
6. Allow the plates to cool and dry, and store at 4°C.

Columbia Agar[1]

Formula

	grams per litre
Special nutrient substrate	23.0
Starch	1.0
Sodium chloride	5.0
Agar	13.0

pH 7.3 ± 0.2

[1] – Merck KGaA, Darmstadt, Germany.

Appendix 3. Preparation of *Campylobacter* Blood-Free Selective Medium (Modified CCDA- Preston)

Steps

1. Suspend 22.75 grams of *Campylobacter* blood– free selective agar base (code CM739) in 500 ml of distilled water and bring to boil to dissolve.
2. Sterilize the suspension by autoclaving at 121°C for 15 minutes.
3. Cool to 50° C.
4. Aseptically add 2 ml of sterile distilled water to one vial of CCDA selective supplement (code SR155E) and mix gently to dissolve.
5. Add the vial contents to the 500 ml *Campylobacter* blood–free selective agar base when it has cooled (see step 3 above)
6. Mix well and pour into sterile petri-dishes.

Campylobacter blood–free selective agar base (CM739)[1]

Formula

	grams per litre
Nutrient broth No.2	25.0
Bacteriological charcoal	4.0
Casein hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0

pH 7.4 ± 0.2

CCDA selective supplement (SR155E)[2]

Vial contents

Cefoperazone	16 mg = 32 mg/ litre of medium
Amphotericin B	5 mg = 10 mg/ litre of medium

[1] – Oxoid Manual 1982/86
[2] – Oxoid limited, Basingstoke, Hampshire, England.

Appendix 4. Preparation of Tryptic Soy Agar (TSA)

Steps

1. Suspend 40.0 grams of Tryptic Soy Agar powder in 1 litre of distilled water and heat to boiling to dissolve completely.
2. Sterilize by autoclaving at 121° C for 15 minutes.
3. Cool to 50° C.
4. Pour into sterile petri-dishes.

Tryptic Soy Agar[1]

Formula

	grams per litre
Tryptone Peptone (Pancreatic digest of casein)	15.0
Soytone Peptone (Papaic digest of soybean meal)	5.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.3 ± 0.2 at 25 ° C

[1] – DIFCO Manual, 10th Edition, Becton Dickinson and Company, USA.

Appendix 5. Preparation of glycerol broth (15%)

Steps

1. Suspend 8.0 grams of nutrient broth powder in 1 litre of distilled water.
2. Add 150 ml of glycerol[1] and mix well.
3. Dispense 3 ml aliquots into bijoux bottles.
4. Sterilize by autoclaving at 121°C for 15 minutes and store at 4°C.

Nutrient broth[2]

Formula

	per litre of medium
Beef extract	3.0g
Peptone	5.0g

Final pH 6.8 ±0.2

[1] – BDH laboratory supplies, Poole, England.
[2]- DIFCO laboratories, Detroit, USA.

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