Detection of *Campylobacter jejuni* in poultry samples using Fluorescent *in situ* Hybridization (FISH)

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Abstract

In this study, a protocol using Fluorescent *in situ* Hybridization (FISH) for the detection of *Campylobacter* spp., particularly *C. jejuni*, in naturally contaminated poultry samples was developed and its application evaluated. The protocol used would eliminate the need for the conventional plating of enriched samples, thereby significantly reducing the time needed to detect the presence of *Campylobacter* spp. Using a fluorescence plate reader, the detection limit for pure *C. jejuni* was 7.33 $\log_{10}$CFU/ml. Several types of enrichment media were tested and Bolton Broth with blood (aerobic and microaerophilic) and Bolton with *Campylobacter* Growth supplement (microaerophilic) were most effective for culturing samples spiked with low levels of *C. jejuni*. Applications of FISH for spiked poultry samples showed that blood will interfere with sample processing and greatly increase non-specific background fluorescence. The use of a pre-treatment step prior to FISH, which included blood lysing, buoyant density centrifugation (BDC) and filtration through a 0.8μm membrane filter with a prefilter reduced background fluorescence. BDC, a simple and rapid centrifugation method, was shown to have a high cell recovery, to increase cell concentrations by 0.6-1.4$\log_{10}$CFU/ml and to remove background blood and chicken particles interfering with FISH. The FISH procedure, used after 48h enrichment with the application of the pre-treatment step was considered to be capable of detecting *Campylobacter* spp. in naturally contaminated poultry samples, although this was dependent upon the enrichment medium.

Microaerophilic enrichment in Bolton Broth with blood or with growth supplement was considered more effective than aerobic enrichment in Bolton Broth with blood, which resulted in fewer positive samples and significantly lower *Campylobacter* cell counts. The ability of FISH to detect *Campylobacter* spp. in naturally contaminated poultry samples was determined after

microaerophilic enrichment in Bolton Broth with *Campylobacter* Growth supplement. Of the 36 samples tested, 23 were positive for both FISH and plating from any of the three enrichment media, but 10 of the 36 samples were positive for FISH only. This discrepancy may be due to background flora either overgrowing *Campylobacter* spp. on the plates or high cell counts of non-*Campylobacter* may cause background fluorescence. The FISH procedure was shown to be effective in detecting *Campylobacter* spp. in poultry samples, although the effect of background flora on sample fluorescence and false-positives should be examined.

1. Literature Review

1.1 Introduction

There are millions of cases of food-borne disease each year throughout the world. Of these incidences, *Campylobacter* is the leading cause in many industrialized countries, including the USA, Canada and United Kingdom. In the USA, there are 2.1 to 2.5 million cases of campylobacteriosis annually, accounting for an average of 46% of all food-borne disease incidences. This represents almost twice as many cases than salmonellosis, and considerably more than shigellosis and infections by *E. coli* O157:H7 (Altekruse et al, 1999).

Campylobacter, compared to other food-borne pathogens, has had a relatively short history. The time of first discovery of the bacterium is unknown, but there are reports of symptoms resembling *Campylobacter* infections dating back to the beginning of the 18th century (Altekruse et al, 1999). Prior to the 1970's, *Campylobacter* had been isolated from animals only and was only considered important in veterinary medicine (Ketley, 1997). To date, 15 species of *Campylobacter* have been isolated and identified and several of them have been found to cause disease in humans (Park 2002). *C. coli* and *C. jejuni* are responsible for more than 80% of the incidences each year and the remaining cases are typically caused by *C. lari, C. fetus, C. upsaliensis* and *C. hyointestinalis* (Ketley, 1997). However, it has been suggested that the contribution of these other *Campylobacter* species to human disease may be underestimated. This may be due to the environmental conditions used for the recovery of the organisms, as there exist species variations for optimal growth temperatures and nutrient requirements. Of all *Campylobacter* species, *C. jejuni* has been researched most extensively because of its economical and health impact.

The focus of this review is to introduce the physiology of *C. jejuni* and the importance of this organism in the poultry industry. An overview of the poultry processing industry will describe modes of transmission, sources of contamination and methods for detecting the presence of *Campylobacter* throughout rearing, processing and packaging.

1.2 Physiology of *C. jejuni*

C. jejuni is a gram-negative, non-spore forming rod. The rod is normally spiralled, but it can become a cocciod body when the cells are old or stressed due to a prolonged exposure to oxygen. They are about 0.5 - 5μm in length and about 0.2 – 0.9μm wide. The organisms possess a single polar flagellum that has been linked to pathogenicity (Wassenaar and Blaser, 1999). *C. jejuni* is commonly associated with birds, in particular poultry. Domestic animals such as cattle, pigs, dogs and cats can be carriers of the organism and outbreaks have been associated with the consumption of non-pasteurized milk or drinking water contaminated with fecal matter.

The growth requirements of *Campylobacter* are representative of the organism's host environment. *Campylobacter* grows optimally at 42-45°C and growth is inhibited below 30°C and above ideal temperatures (Park, 2002). *Campylobacter* is a microaerophilic organism, meaning they only require a small concentration of oxygen. An optimal atmosphere for growth consists of 5% oxygen and 10% carbon dioxide. At oxygen concentrations above 21%, growth can be inhibited, but the organism will not die and may grow again once an optimal atmosphere is reached. *C. jejuni* is considered a fastidious organism due to the strict growth requirements and nutrient factors needed for healthy cells. The most important growth factors required by *Campylobacter* are those capable of sequestering oxygen thereby reducing the presence of toxic compounds that can result from chemical reactions with oxygen. *C. jejuni* is also known to have

a low tolerance for salt, acidity and dry environments, which along with adverse atmospheric and temperature conditions can lead to stress of the cells (Park, 2002).

In stressed environments, *Campylobacter* will enter into a viable but non-culturable state (VBNC) (Tholozan et al, 1999). The term VBNC refers to a physiological state that was first identified in environmental microbiology, and has now been reported for many organisms associated with food. A generally accepted definition for VBNC states that VBNC cells are metabolically active, but can not be cultured on standard culture media (Thozolan et al, 1999). Although VBNC cells are metabolically active, they have reduced cellular functions and do not multiply. The VBNC state is considered a means of protection against adverse environments and has been identified in a number of non-spore forming organisms (Doyle et al, 2001). Factors that induce VBNC include extreme temperature, atmospheric conditions, osmotic pressure and insufficient nutrient sources (Chaveerach et al, 2003). Numerous studies have demonstrated the ability of *C. jejuni* to become VBNC when stressed (Tholozan et al 1999, and Hazeleger et al, 1998). The response of *C. jejuni* to temperature stress and its effect on survival during processing and storage of poultry will be discussed in Section 1.4.5.

1.3 Campylobacteriosis

An infection with *Campylobacter* leads to the disease campylobacteriosis. Studies have shown that the infectious dose of *C. jejuni* can be as low as 500 cells, which is relatively low when compared to the infectious dose of *Salmonella,* which in most cases is 10^5 cells (Nachamkin and Blaser, 2000). Once ingested, the bacteria will colonize the intestinal tract, but the symptoms do not start until about 2 to 10 days later (Nachamkin et al, 2000). The severity of the disease depends on the size of the inoculum, the immune status of the patient and the

Campylobacter species involved. Although *C. jejuni* is responsible for 80-90% of campylobacteriosis cases, *C. coli* causes a large portion of the remaining cases, with *C. lari, C. hypointestinalis* and *C. upsaliensis* representing only a small percentage (Keltey, 1997).

Initial symptoms of campylobacteriosis are abdominal cramping followed immediately by watery diarrhea (Altekruse et al, 1999). Since the cells invade the intestinal lining, some patients will lose bile and blood during the infection, which can mimic the symptoms of appendicitis. Along with diarrhea, a patient can also experience nausea, fever, vomiting, headache and chills. Duration and severity of the infection are greatly increased in individuals that are immunocompromised, but in most patients the symptoms are self-limiting and subside within a week (Wassenaar and Blaser, 1999). In many cases, the presence of *C. jejuni* can still be detected weeks after symptoms have subsided and some individuals suffer relapses, which are typically less severe than the initial disease (Ketley, 1997). This asymptomatic shedding of *C. jejuni* leads to the potential problem of carriers, who can spread the organism to other individuals.

Some sequelae of *C. jejuni* infections can lead to chronic health problems, in particular Guillian-Barré syndrome (GBS), which can occur in one of every 2000 cases of campylobacteroisis (Allos et al, 1998). GBS resulting from an *C. jejuni* infection is an autoimmune disease that leads to acute flaccid paralysis. Evidence suggests that antibodies targeted against *Campylobacter* also attack nerve cells, specifically myelin, which surrounds the cells and is required to send nerve impulses efficiently and rapidly (Nachamkin et al, 1998). It has been shown that some surface lipopolysacchrides (LPS) of *C. jejuni* are homologous to surface epitopes of gangliosides (Nachamkin et al, 1998), the abundant glycolipid of myelin (Alberts et al, 1994). As the gangliosides are destroyed by antibodies produced in response to *C.*

jejuni, the nerve cells demyelinate, which leads to limb weakness, loss of tendon reflexes and autonomic abilities (Hahn, 1998). Other sequelae of *C. jejuni* infections include rheumatologic disorders, arthritis and bacteremia (Nachamkin, 2002).

1.4 *Campylobacter* spp. in poultry production and processing

The presence of *C. jejuni* in poultry is considered a public health issue, as raw or undercooked poultry is the most common source of human infections. Incidences of campylobacteriosis are typically due to the consumption of undercooked poultry meat or the cross-contamination of other foods by unhygienic food-handling (Pearson and Healing, 1992). Raw poultry meat can become contaminated during several steps in the production and processing of broiler hens as outlined in Figure 1.1. Research has begun to focus on reducing or eliminating these microorganisms from the poultry industry by identifying areas of processing that are most likely to cause contamination of poultry carcasses.

1.4.1 Contamination with C. jejuni at the rearing farm

In birds, *C. jejuni* will colonize the ceca and small intestine, although the organism has also been isolated from the spleen, liver and other areas of the intestinal tract (Newell and Fearnley, 2003). A study by Berndtson et al (1992) demonstrated that *C. jejuni* can be found in muscle tissue, but the prevalence was relatively low with only 3%. Once the intestinal tract is colonized, the concentration of *C. jejuni* can reach levels as high as 10^9 CFU per gram of intestinal contents (Stern et al, 2003). Several studies have shown that in birds other than broiler hens, the infection can be self-limiting within only 4 weeks (Newell and Fearnley, 2003). A study by Achen et al (1998) demonstrated that this self-limiting infection can also occur in

broiler hens infected with *C. jejuni.* Eight weeks after infection, the number of cells present in cecal samples decreased and the number of positive hens also decreased. However, broiler hens are slaughtered at about 47 days of age, at a time before the infection would be eliminated from most birds. This means that an infected flock would enter the slaughter plant and be capable of cross-contaminating other *Campylobacter*-negative flocks that are slaughtered at the same time.

C. jejuni is introduced into the slaughter plant by hens that were colonized during rearing at the farm. Studies have shown that flocks entering a plant can range from free of *Campylobacter* to 100% of the flock being infected (Atanassova and Ring, 1999). The epidemiology of the *Campylobacter* spp. and *C. jejuni* has been examined in several European countries. The prevalence of *Campylobacter* spp. in the broiler hen flocks of these countries was mostly around 40%, with Great Britain reading as high as 80% (Table 1.1). From 1994 -1995 the US Department of Agriculture (USDA, 1995) performed a baseline study to determine the prevalence of several pathogenic organisms in poultry, including *C .jejuni* and *C. coli.* The results of this study showed that 82% of broiler carcass rinse samples were contaminated with either *C. jejuni, C. coli* or both (Table 1.1). Currently, there is a lack of recent epidemological studies showing the prevalence of *C. jejuni* in broiler hens for both Canada and the USA (Stern et al, 2003).

C. jejuni can infect birds through vertical or horizontal transmission, although the susceptibility of the hens to the infection is dependent on the age of the bird and the strain of *C. jejuni* (Evans and Sayers, 2000). Vertical transmission is still highly debated as a means of infection, due to conflicting experimental results. This mode of infection refers to the passing of *C. jejuni* from the infected parent birds to the offspring through contaminated eggs (Pearson et

al, 1996), compared to horizontal transmission where the organism is passed through ingestion (van de Giessen et al, 1992).

Pearson et al (1996) used serotyping to compare *C. jejuni* strains in flocks grown from one of two hatcheries and a third that had been supplied hatchlings from both hatcheries. Results of the study showed that several serotypes were linked to each hatchery, but in the flock of mixed hatchlings, only isolates of hatchery B were isolated. It was suggested that if the transmission of *C. jejuni* to the new flock had been horizontal, there would have been an even distribution of isolates from both hatcheries. The authors suggested that vertical transmission contributed to the colonization of hatchlings in the mixed flock, but that horizontal transmission was the cause for the spread of *C. jejuni* within each hatchery. In recent years, there have been numerous studies implicating horizontal transmission as the only route of *C. jejuni* infection, suggesting that the vertical transmission reported by Pearson et al (1996) was actually due to horizontal transmission. Two significant factors have been used to argue against vertical transmission. First, it has been suggested that *Campylobacter*-positive chicks may not have been infected by the parent birds, but instead, through fecal contamination present on the egg-shell. *C. jejuni* cells on the surface of the eggshell can be transferred to the chick during hatching, explaining *C. jejuni* colonization immediately after hatching. Newell and Fearnley (2003) also suggested that cracks in the eggshell may allow the bacterial cells to enter the egg prior to hatching, facilitating the infection of the chick during hatching. It has been demonstrated that colonization of broiler chicks can be delayed for up to two weeks after hatching (Stern and Robach, 2001). This is in stark contrast to the vertical transmission theory where chicks would be born already infected. Two studies by Sahin et al (2001 and 2003) showed that the delay in colonization by *C. jejuni* may be due to the presence of *Campylobacter* specific maternal

antibodies (MAB). In a first study Sahin et al (2001) used the enzyme-linked immunosorbent assay (ELISA) to detect the presence of MAB in chicks and to determine the duration of these positive antibody titres. MABs were detected in 100% of one-day old broiler chicks and they were present for up to 14 days, after which there was a significant drop in MAB concentration. After 2-3 weeks, MABs were no longer detectable, showing that they have a lifespan of only 2 weeks. In a second study, Sahin et al (2003) demonstrated that MABs are capable of delaying the onset of colonization and reducing horizontal transmission amongst young chicks. Although the MABs can protect against infection in younger chicks, their effectiveness is limited, as large doses of *C. jejuni* will overwhelm the antibodies, allowing the colonization of the chick. Experiments showed that significantly fewer MAB-positive 3-day old chicks were infected with *Campylobacter* compared MAB-negative 3-day old chicks (Sahin et al, 2003). The authors also suggested that there may be other unidentified factors that contribute to immunity.

Presence of *Campylobacter* on eggshells is only one of the many factors that contribute to the contamination of broiler flocks. Other sources include water contamination, animal vectors and poor hygiene of the farm environment and its workers (Shreeve et al, 2002). Soil, rodents, wild birds and insects are considered potential reservoirs, as they have been linked to the spread of *Campylobacter* from neighbouring farms (Refregier-Petton et al, 2001). This can explain the presence of more than one strain of *C. jejuni* within a flock. As *C. jejuni* is an enteric organism, it is present in large concentrations in the fecal matter of colonized birds. Once excreted from the host, *C. jejuni* can spread to other areas including the floor, bedding and even other hens (Refregier-Petton et al, 2001). The organisms are typically transferred from the environmental surfaces to the feathers and the cells are subsequently ingested during preening. The focus of many researchers is to eliminate the presence of *C. jejuni* at the farm level, as has been done with

other food pathogens such as *Salmonella*. However, this has been unsuccessful and the current approach is to reduce the opportunities for the organisms to enter into the farm area. This is achieved by maintaining strict hygienic procedures, use of disinfectants and minimizing the traffic in and out of the farm. A study by Shreeve et al (2002) used *fla* typing to examine the effect hygienic methods on the persistence of *C. jejuni* in successive broiler flocks. Results showed a low level of isolate carry-over. It was suggested that cleaning and disinfecting of the area prior to introducing the next flock was effective at reducing the presence of *C. jejuni* or eliminating the cells from the farm. Also, the study found very few sources of *Campylobacter* within the farm area and it was suggested that potential reservoirs could be present outside of the farm (Shreeve et al, 2002).

1.4.2 Transportation of flock to processing plant

Once the flock is ready for slaughter, the birds are crated and placed in trucks for transport to the processing plant. During transportation, fecal matter can contaminate broiler hens through either direct bird to bird contact or through presence of the fecal matter on the transport crates. The risk of cross-contaminating *Campylobacter* is considered low, although a study by Slader et al (2002) demonstrated that fecal material and *C. jejuni* could remain viable on the crates, even after exposure to disinfectant washes. A possible explanation for the survival of *C. jejuni* is the presence of organic compounds in the fecal material that may protect the cells against disinfectants and detergents (Slader et al, 2002). To alleviate some of the problem, the broiler hens are denied food and water up to 12 hours before transport, which reduces the amount of fecal matter carried by the hens (Corry and Atabay, 2001). Once at the factory, the birds are placed in the reception area and prepared for slaughtering.

1.4.3 Defeathering

After the bleeding of the hen by the cutting of the carotoid artery, the next step is to remove the feathers. The defeathering process begins with a scald of the carcass. The birds, hung by the feet, are passed through a water bath set at 50°C to 60°C, which causes the feathers to loosen from the skin, making them easier to pluck. Once scalded, the carcass is sent through a defeathering machine, where the feathers are beaten off using strips of rubber. Scalding and defeathering are the first stages of poultry processing that are considered a high risk of cross-contamination by *Campylobacter*. The *C. jejuni* cells present on the feathers are capable of contaminating the scald water, which can lead to contamination of subsequent carcasses passing through the water. Berndtson et al (1992) suggested that *C. jejuni* can enter the subcutaneous layer of the skin through the feather follicles that open when exposed to the hot water. Although the water is at a high temperature, it is not normally lethal to *C. jejuni*. This problem cannot be eliminated by increasing the temperature, as this would cause the surface of the carcass to discolour, decreasing its value. Chlorinated water that is constantly circulated in a counter-flow system, is a current method that has been implemented in some processing plants to reduce contaminations.

The defeathering strips are also capable of spreading *C. jejuni* to other carcasses. The bacteria present on the feathers can transfer to the rubber strips and then to carcasses passing afterwards. Therefore, the strips need to be disinfected regularly, as the micro-organisms are able to remain viable for significant periods of time.

1.4.4 Evisceration

Of all poultry processing steps, evisceration is considered the most significant factor in the spread of *Campylobacter* (Chantarapanont et al, 2003). During this stage, the stomach, intestinal tract and all other contents are removed from the body cavity. As the gastrointestinal tract of the bird contains the highest concentration of *C. jejuni*, there is a greater chance for contamination of the carcass surface or for cross-contamination of subsequent carcasses (Berrang et al, 2004). Berrang et al (2004) showed the effect of fecal contamination on cell counts of *Campylobacter* on poultry carcasses. This was done by comparing cell counts of *Campylobacter* on broiler carcass halves with and without the addition of cecal contents. The authors transferred 2mg, 5mg, and 10mg of cecal contents to the carcass surface, followed by a carcass rinse to determine the mean number of *Campylobacter* cells present. There was no significant difference in cell counts at low levels of fecal contamination, but at higher contamination levels significant increases in the number of *Campylobacter* were seen. At 5mg of fecal contents, mean bacterial numbers increased from 2.6 $\log_{10}$ to 3.3 $\log_{10}$, a difference of 0.6 $\log_{10}$. At the highest level of fecal contamination (10mg), the mean bacterial counts increased by a full $\log_{10}$, demonstrating that fecal matter does significantly contribute to the level of *Campylobacter* present on carcass surfaces. During processing, any visible fecal matter is removed as a means of reducing the possibility of carcass contamination. After evisceration, a series of wash steps are used to clean the carcass before chilling and further processing or packaging. Berrang et al (2004) suggested that these steps may help to reduce the presence of invisible fecal matter.

1.4.5 Chilling

In processing today, two different systems are used to chill the carcasses. In the older, more traditional method, the carcass passes through an ice-cold water bath. The problem associated with this is similar to the scalding process, where the water can become contaminated and constant water circulation and disinfectant are therefore required. The other method used by some plants is to chill the carcass with air-cooling systems that blast cold air at relatively high humidity (Ellerbroek, 1997). The benefits of this method are that the carcass is not immersed in water possibly contaminated with *C. jejuni* and that the carcass does not have to dry before packaging.

Although *C. jejuni* is considered a thermophilic organism, it has the ability to survive at low temperatures and the chilling process will therefore not kill the bacteria (Lázaro et al, 1999). The effect of low temperature on growth rate and survival of *C. jejuni* was examined by Hazeleger et al (1998). Sample plating was used to determine culturability and tests were performed showing changes in respiration, ATP synthesis and catalase activities. Cells were not culturable below 37°C, but protein synthesis and respiration were detected at temperatures as low as 4°C. This demonstrated that although cells had reduced physiological activities, non-culturable cells could remain viable in environments of suboptimal temperatures. This ability to survive refrigerated temperatures adds to the difficulty of controlling the spread of *Campylobacter* to the consumer and other foods (Chan et al, 2001).

1.4.6 Decontamination of final carcass

The poultry industry has implemented a number of decontamination methods as a means of controlling the prevalence of *Campylobacter,* including the use of chlorinated wash water,

surface heating and also the use of chemicals such as lactic acid (Farkas, 1998). To date it has not been possible to ensure a *Campylobacter*-free product irrespective of the decontamination steps used or the hygiene level present in the processing plant.

1.5 Detection of *C. jejuni* in poultry

Due to the high incidence of *C. jejuni* in poultry, the industry tests for its presence both during the processing of the broilers and on the final product. This allows for the identification of possible areas of contamination and to ensure that levels of *C. jejuni* are within an acceptable range. Due to the difficulties in recovering and culturing *C. jejuni,* detection methods can be challenging and new techniques are being developed. Many of the new methods use molecular-based techniques such as poylmerase chain reaction (PCR), immunological methods, and Fluorescent *in situ* Hybridization (FISH).

A factor that should be considered for all testing methods is the type of sample used to evaluate *C. jejuni* contamination of raw poultry (Jørgensen et al, 2002). There has been no consensus worldwide as to which sample type is most representative of carcass contamination. In general, samples are either a whole-carcass rinse or a portion of meat cut from the carcass. The current sampling approach for both Canada (Mereiros and Hofmann, 2002) and the USA (Hunt et al, 1998) uses a 25g sample cut from the poultry carcass, but it is not stipulated from which area the samples should be taken. A study by Jørgensen et al (2002) compared the effect of sample types on the isolation of *Salmonella* and *Campylobacter* spp. from retail chicken. The samples compared were a whole-carcass rinse, a portion of neck skin and a whole-carcass rinse with the neck skin present. The detection of *Campylobacter* spp. was not dependent on the type of sample used as for the case of *Salmonella* detection. However, a whole-carcass rinse with the

neck skin present had a greater probability of *C. jejuni* detection, compared to using neck skin only (Jørgensen et al, 2002).

1.5.1 Conventional culture procedure

Although the presence of *Campylobacter* on poultry can be determined using a number of novel techniques, the industry continues to use the conventional culture based method. This method, as outlined in Figure 1.2, consists of three main parts; the enrichment of a sample, plating of the sample to isolate suspect colonies and a series of biochemical tests to confirm identity of suspect colonies (Baylis et al, 2000).

The first stage in the process is the enrichment in a medium that is selective for *Campylobacter*. All media described to date rely on the use of antibiotics that the organism is known to be resistant to. This selectivity is required to inhibit or reduce the growth of unwanted background organisms that may out-compete *Campylobacter*. In addition, the medium generally contains a nutrient rich supplement, which also serves to reduce oxygen levels (Blais and Phillippe, 1999). These supplements are either laked blood or a combination of chemicals including ferrous sulphate, sodium metabisulphite and sodium pyruvate (Blais and Phillippe, 1999). The most commonly used base-media are Bolton Broth recommended by the US government (Hunt et al, 1998) and Park and Sanders enrichment broth, used in Canada (Medeiros and Hofmann, 2002). The main disadvantage of the conventional method for the detection of *C. jejuni* is the time required until a result is obtained. Enrichment normally requires a two day incubation, and a study by Engberg et al (2000) demonstrated that *C. jejuni* can take up to seven days to grow to detectable levels if stressed cells are present. Next, the enriched sample is plated on selective media. Like for the enrichment medium, selectivity is

achieved by antibiotics, but they are generally different from those used in the enrichment broth. It may take up to two days until suspect *Campylobacter* colonies can be identified for further testing. The final step of the conventional method includes a series of biochemical tests to confirm that suspect colonies are *C. jejuni* as there are other micro-organism capable of growing in the presence of the antibiotics. The confirmation tests used by both the Canadian and USA poultry industries are similar (Table 1.2). The majority of *Campylobacter* spp. are motile gram-negative curved rods which are oxidase, catalase, and nitrate reduction positive, but negative for glucose fermentation. Susceptibility to cephalothin and nalidixic acid is used to separate different *Campylobacter* spp. *C. jejuni* can be distinguished from all other *Campylobacter* spp. by the ability for hippurate hydrolysis. Identification with biochemical testing can be limited as mutations in the genome or changes in physiological state can affect whether an organism will consistently produce the same characteristic set of results.

Generation of the microaerophilic atmosphere required for the growth of *C. jejuni* can be expensive. Currently, the atmosphere can only be created by either using a gas incubator, which is constantly supplied with gas from cylinders or by placing gas packs in a sealed jar, where a chemical reaction creates the atmosphere. A disadvantage to generating the atmosphere with chemical reactions is that it is maintained only as long as the jar is sealed, unlike gas incubators. However, a study by Van Horne et al (1999) showed that although gas packs can only be used once, their use is significantly less expensive compared to an incubator. A more cost efficient means of incubating *C. jejuni* would be beneficial, that would eliminate the need for an microaerophilic atmosphere. Bolton Broth, the standard medium recommended by the USDA is currently used microaerophilically, but it has been shown to support the growth of *C. jejuni* aerobically (Oxoid Ltd.). However, Blais et al (1999) reported that the medium could not

support the enrichment of *C. jejuni* to high cell concentrations, compared to the same medium incubated microaerophilically. The numerous disadvantages of the conventional detection method, have lead to the development of alternative techniques that would no longer depend on culturability and phenotypic traits. These include immunological methods, the polymerase chain reaction (PCR) and fluorescent *in situ* hybridization. The sensitivity of these methods depends on the number of organism present, therefore initial pre-enrichment is still desirable and can not be completely excluded from a protocol

1.5.2 Immunological methods

The term "immunological" refers to methods that use immunogenic properties of the cells to identify the species of microbe present. The most common method uses antibodies that bind to a specific surface protein of the target organism. Examples of two methods designed to detect the presence of *C. jejuni* are the Enzyme-Linked Immunosorbent Assay (ELISA) and the colony-lift immunoassay (CLI). Although both methods use an enrichment step, results are still obtained in less time than with enrichment and plating onto selective media.

Lilja and Hanninen (2001) used ELISA to test for the presence of thermophilic *Campylobacter* species on chicken samples. The procedure used fluorescent antibodies that, once bound to the cells, could be detected by means of the EiaFoss Campylobacter System. An enrichment step prior to ELISA was necessary for optimal results and a final result could be obtained within 2-5 days (Lilja and Hanninen, 2001).

Not only does CLI identify a target organism, it can give an estimate of cell numbers present in the original sample. Rice et al (1996) tested CLI for the detection and enumeration of *C. jejuni, C. lari* and *C. coli* and optimized the procedure. The samples were grown on selective

media or filters and incubated until colonies appeared. The colonies were lifted from the agar or filter and then subjected to a series of steps that allowed the antibodies to bind to the cells. This resulted in a membrane with fluorescing target colonies. The selective medium used in the experiment still allowed for the growth of non-target microbes, which sometimes overgrew *C. jejuni* colonies. The benefit suggested by Rice et al (1996) is that the procedure is relatively simple and appears not to be inhibited by the presence of food particles.

1.5.3 Detection with PCR

In recent years, the polymerase chain reaction (PCR) has been used extensively as a means of identifying the presence of specific microorganisms. The principle behind a PCR is to create a large number of copies of a specific nucleotide sequence. A PCR is performed in three stages; denaturing, annealing, and extension. In the denaturing step, high temperatures are used to separate the strands of DNA into single strands. The temperature is dropped leading to the annealing stage in which primers bind to the complementary DNA strands. During the final extension stage, the DNA strand is replicated using the bound primer as an initiation site. These steps are continued through 20-30 cycles producing a 10^6 to 10^9 fold increase in the amount of initial target DNA. There are a number of variations of PCR that have been used to detect and/or identify *Campylobacter*, including nested PCR, semi-nested PCR, multiplex PCR and nucleic acid sequence-based amplification (NASBA). The primers used must be species specific to genes only present in *C. jejuni*. Commonly used primers for *C. jejuni* include the flagella genes *fla*A and *fla*B and in some cases the conserved sequences of the region coding for the serine hydroxymethyltransferase, *glyA* (Nachamkin and Blaser, 2000).

A common problem associated with PCR is the sensitivity of the procedure in relation to the presence of background particles and chemicals. This is generally not of concern when working with pure cultures diluted in buffer, but it may be a limiting factor when testing industry samples, including those from waste treatment plants and the food industry. Analysis of poultry can be problematic when using PCR, as meat particles are known to interfere with several stages of the procedure. Methods have been developed to remove such inhibitory particles, in particular buoyant density centrifugation, which will be discussed in Section 1.7.

1.5.3.1 Nested PCR

Nested PCR consists of two PCRs performed in sequence. The first PCR amplifies a nucleotide sequence from the organism's complete DNA using two primers that are termed 'outer primers'. The product from the first PCR reaction serves as the target DNA for the second PCR and another set of 'inner primers' will amplify a smaller portion of target DNA (Winters et al 1997). This eliminates DNA products that were amplified due to non-specific binding during the first round, allowing for more sensitive and precise results. A study performed by Winters et al (1997) showed that nested PCR could be used to detect *C. jejuni* in chicken rinses, with the use of only a single preparation step. Amplification from non-specific binding was significantly reduced and the final procedure was capable to detect concentrations of *C. jejuni* as low as 10^2 CFU/ ml within only one day (Winters et al 1997).

1.5.3.2. Semi-nested PCR

The principle of semi-nested PCR is similar to nested PCR, in that a second PCR is performed. In semi-nested PCR, only one primer from the first PCR is replaced for the second

PCR, unlike in nested PCR where both primers are replaced. Wagge et al (1999) used semi-nested PCR to identify the presence of *C. coli* and *C. jejuni* in sewage, water samples and food samples. The two species could be distinguished based on the size of the PCR product, amplified from the intergenic sequence between the *fla*A and *fla*B genes (Wagge et al, 1999). With the addition of a non-selective enrichment step, the detection limit was as low as 30 to 150 CFU/ L of water (Wagge et al, 1999). The enrichment broth was non-specific and was incubated in conditions that would allow the VBNC to recover and grow. The enrichment of the cells and centrifugation of the sample allowed the researchers to remove the inhibitory substances, particularly the humic compounds found in the water samples.

1.5.3.3 Nucleic acid sequence-based amplification (NASBA)

NASBA is a deviation of a traditional PCR, in that RNA is amplified instead of DNA. The reaction requires RNA polymerase, RNase H and avian myeloblastosis virus reverse transcriptase (Uyttendale et al, 1995). The addition of complementary primers allows the target RNA to be transcribed into double-stranded DNA. Using a second set of primers the RNA polymerase replicates thousands of copies of the RNA (Cook, 2003). Uyttendale et al (1995) performed NASBA with several foods to detect *C. jejuni*. For all the foods, except milk, the detection limit could be as low as 10 CFU/ 10 g of food. In contrast to PCR, the NASBA was not inhibited by any particles present in the sample (Uyttendaele et al 1995). When performing NASBA, it is more likely that the primers will find strands of RNA to bind to, because of the large number of RNA copies found within one cell; whereas, when using PCR, there is only set of genomic DNA present.

1.6 Fluorescent in situ Hybridization

Fluorescent *in situ* Hybridization (FISH) has been recently introduced as a new approach for identifying the presence of microbes. FISH uses fluorescently labelled molecular probes that emit a distinct wavelength of light when excited with a specific wavelength of light. This procedure is based on the *in situ* hybridization (ISH) designed in 1969 by two separate research groups, but it was not until 1988, that this procedure was first implemented in microbiology (Moter and Göbel, 2000).

Traditionally, this technique has been used for microbial ecology as a means of studying bacterial diversity in a variety of environmental samples (Amann et al, 1997). There are now studies published showing that FISH can be used to detect specific bacteria present in blood, food products and water treatment plants. The ability of this method to detect and identify bacteria effectively is dependent on the probe sequence used.

1.6.1 Molecular probes

FISH is performed using molecular probes designed to bind to the organism's ribosomal RNA (rRNA) sequences. Ribosomes are organelles found in large quantities in every living cell, which are responsible for the translation of RNA into proteins necessary for the functioning of the cell. The rRNA molecules have both structural and catalytic functions within the ribosome, being an integral part of protein synthesis (Madigan et al, 2000). In a normal healthy cell, there are anywhere from 10^2 to 10^5 ribosomes and each ribosome consists of two subunits, each with their own rRNA (Coşkuner, 2002). The large subunit, also called the 23S subunit, has a rRNA strand of about 3000 nucleotides in comparison to the small subunit, known as the 16S subunit, which consists of about 1,500 nucleotides (Amann et al, 1997). The 16S subunit is most often

used as target for FISH, as it is generally employed in phylogenetic studies and has been sequenced for a wide variety of organisms (Amann et al, 1997). The use of rRNA was based on evidence showing that rRNA sequences are highly conserved within a species, but also posses highly variable regions, which vary for different species. This makes rRNA ideal for designing a probe than would be specific for a given species of bacteria. The target sequence chosen for FISH is not only dependent on how conserved the area is, but also on accessibility to the probe. In some instances, the target area can be inaccessible to the probe, due to interactions with ribosome proteins or secondary structures of the rRNA strands. Such interference with FISH can be reduced or eliminated by the use of chemicals, such as formamide, which are added to the hybridization buffer (Coşkuner, 2002).

There are two general classes of molecular probes available for FISH, oligionucleotide and polynucleotides probes. Oligionucleotides probes are those that have less than 20 nucleotides, whereas polynucleotide probes contain a greater number of nucleotides (Amann et al 1997). Oligionucleotide probes are most commonly used, because they are less expensive to produce and it has been found that specificity is sometimes compromised with increasing number of nucleotides (Glockner et al, 1996). The rRNA sequences of strains from a single species may possess single nucleotide variations. These variations affect hybridization with long probes more than with short probes, as the longer the sequence, the more likely it is that these mutations will interfere with the probe binding to a complementary strand of rRNA (Amann et al, 1997). During the FISH procedure, cells are permeabilized with chemicals such as alcohol to allow the probes to freely pass through the plasma membrane. If the probe is too long or bulky, it will not be able to pass through the plasma membrane and therefore hybridization cannot occur (Coşkuner, 2002).

There are a number of fluorescent dyes available, such as fluorescein and rhodamine, which can be attached directly to the probe (Amann et al 1997). In general, these fluorescent labels do not affect probe hybridization unless they are too large and impede passage of the probe through the plasma membrane.

1.6.2 Benefits of FISH

FISH is considered a better detection method compared to the traditional culturing approach for several reasons. Traditional methods involve enrichment and plate culturing, followed by a series of biochemical tests. The probe sequence is designed based on rRNA sequences that are relatively well conserved, unlike biochemical tests that can be different for organisms of the same species because of phenotypic variations (Fang et al, 2003). Detection of *C. jejuni* by enrichment can take up to five days because of the slow growth of this microbe. Economically, FISH can be less expensive with the need for less materials and time, as it can give a positive result by several days earlier than traditional methods, as enrichment and the FISH procedure require a total of three days to perform (Fang et al, 2003). Selective media will only indicate the presence of *Campylobacter* spp., but not which species was found in the sample. When performing FISH, species specific probes can be used and the bacteria identified to the species level.

Several studies have compared FISH and PCR, including the detection of *Campylobacter* in poultry samples (Moreno et al, 2001) and the detection of *Arcobacter* and *Campylobacter* strains in sewage samples (Moreno et al, 2003). The results of these studies suggest several benefits and disadvantages, which will be discussed in further detail in Chapter 3. The two main advantages to FISH are that the procedure is less prone than PCR to the presence of inhibitory

particles and that RNA instead of DNA is the target. When bacterial cells die, the RNA is quickly degraded and are no longer detectable. DNA however can remain present in a sample for long periods of time. DNA that remains in the sample can be detected by PCR and therefore would cause a positive result even through no viable cells are present.

1.6.3 Limitations of FISH

Potential problems with the FISH procedure have been found when studying microbial ecology. The presence of autofluorescent bacteria may sometimes give false results, as the background fluorescence can be read as a positive probe signal, even if no target organisms were present in the sample. This can be avoided by bleaching the cells or adding formaldehyde prior to the hybridization procedure. A more effective solution is to change the fluorescent dye used to label the probe (Coşkuner, 2002). By choosing a label with emission wavelengths above or below the wavelength of the autofluorescence, the background fluorescence should no longer be detected.

Although FISH has been shown to detect the presence of VBNC cells, this can also be a factor in effectiveness of the detection. Cells that are injured or have a reduced metabolism can have relatively low amounts of RNA present (Fang et al, 2003). This can lead to a lower probe signal than in healthy, metabolically active cells or to no signal being detected (Coşkuner, 2002). There are studies showing that enrichment can allow the cells to repair or become metabolically active and therefore greatly increase the amount of RNA present in each cell. However, the length of incubation must be minimal, because over extended incubation times, the cells will begin to die and the RNA is quickly degraded (Fang et al, 2003).

1.7 Buoyant Density Centrifugation

Buoyant Density Centrifugation (BDC) has been used for a variety of purposes, the most common are the enrichment and purification of a cell culture and the removal of particles that may be inhibitory to new sensitive detection methods. The BDC method uses a gradient medium allowing particles and cells to move through centrifugation tubes, becoming separated according to their physiological buoyant density. Several variations of BDC have been described, including a variety of media, types of gradients and a simplified method designed by Lindqvist (1997).

BDC has been tested with a number of cell types, including bacteria and tissue cells such as liver, lymphocytes, Kuffer cells and pancreatic cells. There are also studies in which viruses and cellular organelles have been isolated using BDC (Pertoft 2000).

1.7.1 Media for BDC

The type of medium used in the procedure is important, as it should not affect the integrity or normal functioning of the cell. Commonly used media include sucrose, salts, iodinated compounds and commercially made media (Pertoft 2000).

Sucrose was one of the first media used for BDC, but studies have shown that the low molecular weight of sucrose can result in osmotic stress (Xue et al, 2000). This occurs because the small molecules are able to pass through the plasma membrane, causing the cells to shrink due to loss of water (Awasthi et al, 2001). Xue et al (2000) suggested that although sucrose medium is able to separate particles, it is not ideal for studies in which cells must remain viable. Salt media such as CsCl, K-tartate, and NaBr are other examples of low molecular weight gradient media that may affect cell physiology due to osmotic pressure. These media are still

used for experiments in which these problems are not considered to be significant factor (Pertoft, 2000).

Ficoll is a commercial gradient consisting of polymers of sucrose. These polymers give Ficoll a large molecular weight of 400,000, which prevents passage through the plasma membrane. Other commercially made gradients include, iodinated media such as Hypaque, Metrizoate, Metrizamide, Nycodenz and Optiprep. They were originally used as x-ray contrast media, but have been applied to produce gradients similar to Ficoll. These compounds have a low molecular weight and are capable of passing through the plasma membrane, altering the buoyant density. To reduce this problem, the iodinated compounds can be chemically combined with media such as Ficoll to produce a larger molecule (Pertoft 2000).

Percoll was first developed in 1977 as a trademark of Amersham Pharmica Biotech and is used to separate a variety of cell types including bacteria, cellular organelles and tissue cells (Pertoft 2000). Percoll consists of colloidal silica coated with polyvinylpyrrolidone (PVP). The PVP protects the cells against the toxicity of the silica which, by itself, can permanently damage cells. Like Ficoll, Percoll is used with viable cells as it is non-toxic and does not influence the osmotic pressure. One disadvantage is that a salt solution must be added to produce the desired gradient. After adding salt, the mixture can no longer be autoclaved, meaning that all solutions need to be sterilized separately. Suresh and Rehg (1996) performed a study in which BDC, dialysis and glass bead separation were compared as a method of removing unwanted background from samples containing *Cryptosporidium parvum.* Although Percoll had a lower cell recovery than the glass bead and dialysis methods, Percoll achieved a greater percentage of viable cells.

1.7.2 Forming the gradient and adding sample to gradient medium

There are two types of gradients, discontinuous and continuous, that can be used with BDC. Discontinuous gradients are produced by layering varying concentrations of medium on top of each other. During centrifugation, the cells pass through the layers of medium and remain at a level equal to their buoyant density. However, when cells and particles are centrifuged in a discontinuous gradient, concentrations of medium may be pulled down into the layer below, forming a continuous gradient instead.

Continuous gradients are most commonly used for BDC. They can be formed by passing varying concentrations of medium through a gradient-mixing device or by freezing and thawing the solution. The latter approach is not ideal for bacteria or other cells that can be temperature sensitive. Xue et al (2000) used a continuous gradient to separate the haemocytes of the European flat oysters into the three cell types, granulocytes, small and large hyalinocytes. Two separate BDC procedures were required to achieve this. The first BDC used a continuous gradient of Percoll that resulted in two bands, one band consisted of the granulocytes, while the other band contained both types of hyalinocytes. Percoll was unable to separate large and small hyalinocytes. When the band was then sent through a second BDC that used Ficoll, three bands were obtained; one contained pure large hylinocytes, the second inlcuded small hyalinocytes and the third contained a mixture of the two types. The procedure did not affect the viability of the cells as there was no significant difference between the cells that had not gone through the BDC and those that were collected after BDC.

There are a number of ways that the sample and gradient can be placed together in a centrifuge tube, and each method has a different effect on the separation of cells. The most common way of preparing the centrifuge tubes is to place the medium at the bottom of the tube

and layering of sample over top. Pertoft (2000) suggested that this method prevents large debris from contaminating the medium, as it will remain at the top of the gradient while the cells and subcellular particles are separated as they pass through the gradient medium. When the tubes are centrifuged, the sample moves outward toward the bottom of the tube with the densest material settling at or near the bottom of the gradient. If the particles are less dense than the gradient medium, they remain at the top since they will not be able to move through the medium. There are two other methods of preparing centrifuge tubes, which are used less frequently. Either, the sample is mixed with the gradient medium or it is layered underneath a preformed gradient. Once gradient media and sample are prepared, the tubes are spun horizontally, such that that the various cells and particles move to the appropriate density level in the medium.

1.7.3 Variations of BDC

Lindqvist (1997) designed a BDC procedure that uses neither a continuous nor a discontinuous gradient. Instead, the concentration of the medium is selected to allow the target cells to migrate through and concentrate at the bottom of the tube, while the unwanted material with a lower density remains at the top of the medium. Commonly used media for this type of BDC are Percoll and BacXtractor, which is a commercial product similar to Percoll. This method is different from the traditional BDC in that all target cells are spun to the bottom of the tube and not separated into distinct bands. This variation of BDC will be discussed in further detail in chapter 2.

1.7.4 Limitations of BDC

Limitations to BDC include the effect of the gradient medium on cell integrity and the ability of the medium to efficiently separate the cells from background particles. Uyttendale et al (1999) showed that contamination from certain food products, such as soft cheese, are not completely removed by the method described by Lindqvist (1997). Several studies have shown that foods with high concentrations of fats and proteins can move through the gradient along with the target cells. This occurs when the buoyant density of the food particles is similar to that of the target bacteria.

1.8 Thesis Objectives

The objective of this thesis is to use the molecular technique, Fluorescent *in situ* Hybridization (FISH), to design and evaluate a protocol for the detection of *C. jejuni* in poultry. This involves three steps. First, Buoyant Density Centrifugation (BDC) will be examined. Although FISH is less sensitive to inhibitory substances from food than PCR, a sample preparation step prior to FISH, such as BDC would be beneficial, provided that minimal or no cell loss occurs. BDC has been used in previous studies to remove inhibitory particles and to concentrate cells, allowing the enhanced detection of microorganisms. BDC will be examined to determine cell recovery, cell concentrations and effect of temperature on the recovery of cells. Second, a previously designed probe, targeting *Campylobacter* spp. will be used to develop, evaluate and optimize a FISH protocol with respect to specificity and detection limit for pure cultures present in various media, including enrichment media spiked with *C. jejuni* and chicken meat. Third, the optimized FISH protocol will be evaluated using naturally contaminated poultry samples collected in a poultry slaughtering plant.

Table 1.1. Prevalence of *Campylobacter* spp. and the percentage of *Campylobacter* positive isolates identified as *C. jejuni*.

Country	No. of Flocks	No. of Birds sampled	% of *Campylobacter*-positive samples	Percentage of *Campylobacter* samples with *C. jejuni*	Reference
Denmark	8911	89,110	42.5	86.4	Wedderkopp et al, 2001
Great Britain	100	1600	81.6	88.8	Evans and Sayers, 2000
Germany	12	509	45.9	~ 100	Atanassova and Ring, 1999
France	75	375	42.1	91	Refrégier-Petton et al, 2001
Russia	ND	370	31.3	ND	Stern et al, 2004
USA	ND	1297	88.2	ND	USDA, 1995

ND = not done

Table 1.2 Typical biochemical profile of different *Campylobacter* species commonly found in poultry.

Test	*C. jejuni*	*C. coli*	*C. lari*	Other *Campylobacter* spp.
Gram stain	-	-	-	-
motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	-	-	-	-
Nitrate Reduction	+ *	+	+	+
Glucose Utilization	-	-	-	-
Resistance to Nalidixic acid	R	R	R	R
Resistance to Cephalothin	S	S	R	S
Hippurate Hydrolysis	+	-	-	-

* = *C. jejuni* subspecies ***doylei*** is negative
R = resistant
S = susceptible

Figure 1.1. *Campylobacter* spp. contamination during poultry production and processing.

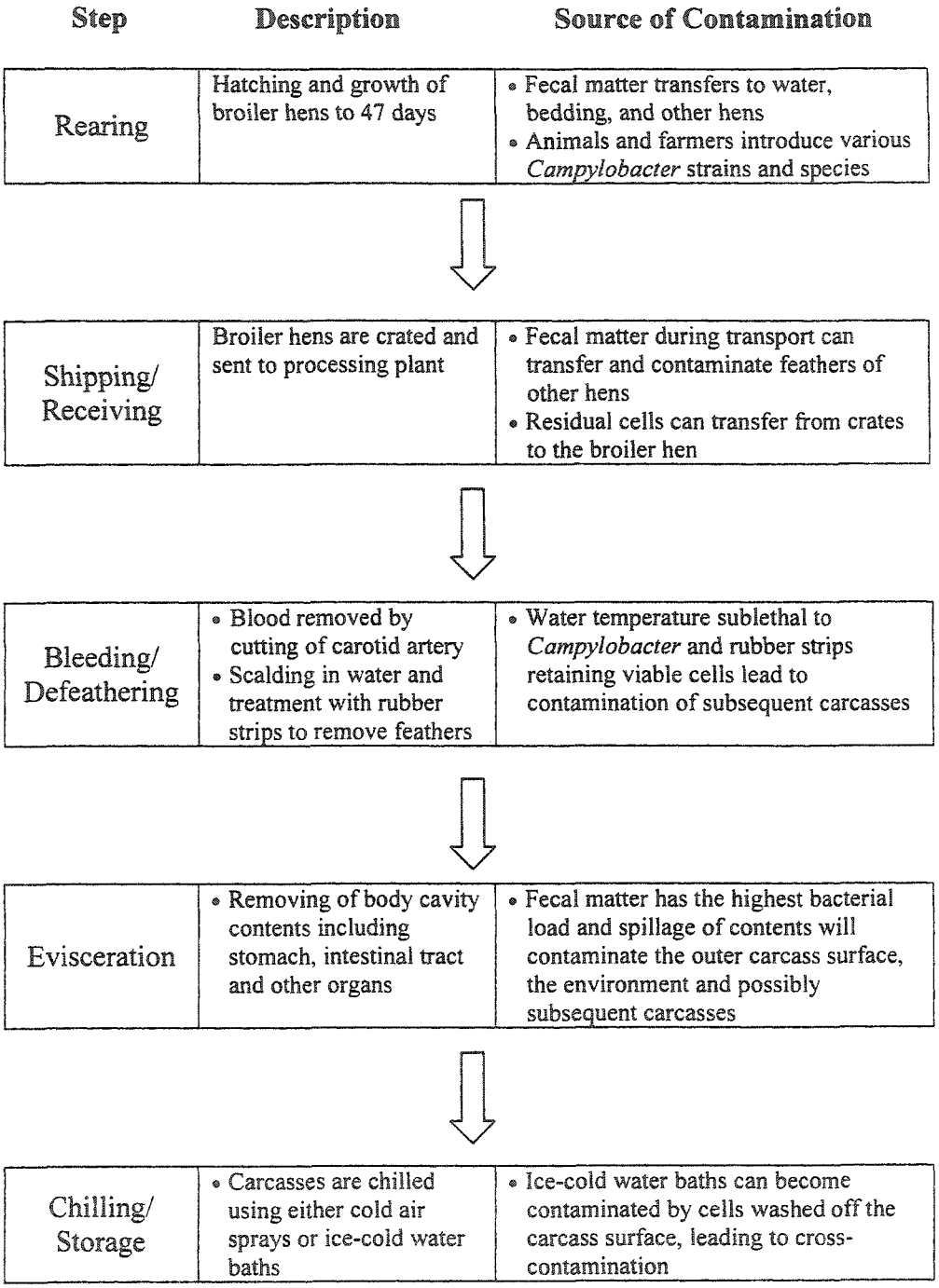
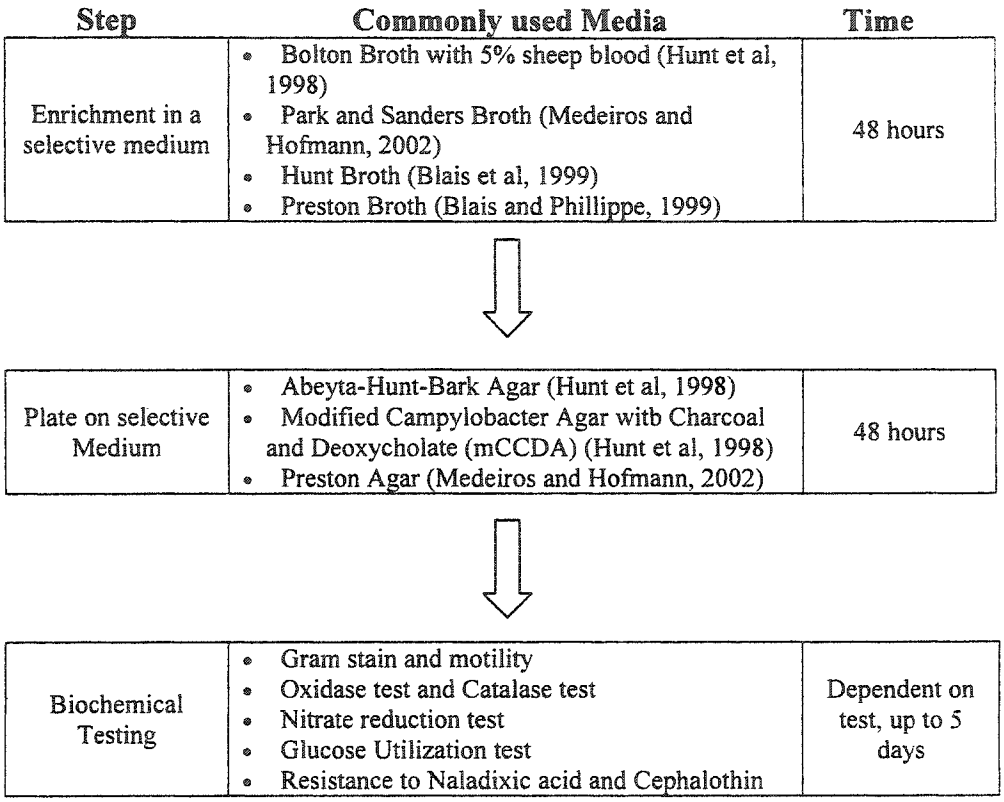


Figure 1.2 Culture based detection of *Campylobacter* spp. in poultry.



Step	Commonly used Media	Time
Enrichment in a selective medium	• Bolton Broth with 5% sheep blood (Hunt et al, 1998) • Park and Sanders Broth (Medeiros and Hofmann, 2002) • Hunt Broth (Blais et al, 1999) • Preston Broth (Blais and Phillippe, 1999)	48 hours
Plate on selective Medium	• Abeyta-Hunt-Bark Agar (Hunt et al, 1998) • Modified Campylobacter Agar witb Charcoal and Deoxycholate (mCCDA) (Hunt et al, 1998) • Preston Agar (Medeiros and Hofmann, 2002)	48 hours
Biochemical Testing	• Gram stain and motility • Oxidase test and Catalase test • Nitrate reduction test • Glucose Utilization test • Resistance to Naladixic acid and Cephalothin	Dependent on test, up to 5 days

2. Evaluation of buoyant density centrifugation for sample purification

2.1 Introduction

Buoyant density centrifugation (BDC), as described in chapter 1, has been used for the separation and concentration of bacteria, viruses and subcellular particles based on their buoyant density. The most common application of BDC is the removal of enrichment medium and sample particles that may interfere with further testing methods, in particular those molecular-based techniques that are sensitive to background matter. It has been shown that by removing background particles, the sensitivity of these procedures can be significantly increased (Uyttendaele et al, 1999).

The traditional BDC protocol uses a large volume to form a gradient that allows for the separation of particles and cells into distinct bands. These large volumes can be limiting as they increase the duration of the spinning and a larger rotor is required for centrifuging, therefore reducing the number of samples that can be processed simultaneously (Lindqvist, 1997). An alternative to the traditional BDC method was developed by Lindqvist (1997). It was designed to eliminate the problems associated with the large BDC volumes, while still maintaining the ability to separate particles from bacterial cells.

Lindqvist (1997) evaluated the effectiveness of this BDC variation at removing particles that may inhibit PCR, using *Escherichia coli* as a target organism. Several types of food homogenates spiked with known levels of *E. coli* were tested, including beef, milk, lettuce, shrimp and two types of cheese. It was determined that food particles with a buoyant density of about 1.035 g/ml would not migrate through the gradient medium as far as the *E. coli* with a buoyant density of 1.051 g/ml, since denser particles will always migrate further down the tube

(Lindqvist, 1997). The focus of the study was to increase the number of samples that could be processed at once. As it was not necessary to separate individual food particles, but to only remove the food homogenate from the bacterial cells, the volume of the density gradient was reduced and a smaller rotor could be used. The gradient was neither continuous nor discontinuous, but instead it was designed so that the cells, which are denser than the gradient, would migrate completely through, while the less dense food particles would remain at the top. Thus, the gradient could be reduced to a final volume of 0.6ml with a sample volume of 0.9ml. The smaller rotor size also reduced the length of centrifugation to a one-minute spin followed by a single wash step. The efficiency of the procedure was evaluated by comparing changes in the detection limit of the PCR targeting *E. coli*. A lower detection limit was observed and this was attributed to the removal of the PCR inhibitors and the concentration of cells that occurs when the sample volume is reduced from 900µl to 10µl. Of the 7 types of food homogenates tested, the soft cheese was the only sample type that could not be separated from the bacterial cells. It was suggested that the cheese particles were of equal density as the bacterial cells and therefore both the food and the cells moved completely through the gradient.

This procedure has since been used for a variety of studies including the separation of *C. jejuni* from food samples prior to further testing. A study by Uyttendaele et al (1999) evaluated the efficiency of BDC for the removal of inhibitors of NASBA-enzyme-linked gel assay (ELGA), using *C. jejuni* as a target bacterium. Similar to findings of Lindqvist (1997), the buoyant density of the food homogenates was 1.033 g/ml, which was lower than *C. jejuni* with a density of 1.084-1.087 g/ml. The BDC procedure was tested with both milk samples and chicken skin samples to evaluate cell recovery and the effect on the removal of NASBA inhibitors. The study concluded that the BDC procedure is an ideal preparation step due to the

simplicity of the procedure, short time requirements and the high cell recovery, in terms of both cell numbers and cell concentration. Wang et al (1999) used this variation of BDC as a means of removing particles that may interfere with the detection of *C. jejuni* in a number of food samples using PCR. The results showed that the method not only increased the detection limit by 10-100 fold, but the PCR signal was considerably more clear than for samples not treated with BDC.

To date, only limited studies have been published that quantitatively examine cell recovery after BDC. The BDC procedure has only been tested indirectly by recording effects on the detection limit of PCR and other molecular-based techniques. The objective of this study was to quantify effects of BDC on cell recovery and cell concentration, using both *Pseudomonas putida* and *C. jejuni* as model organisms. Effects of temperature and suspension medium (buffer, enrichment medium containing blood and chicken homogenate) were also investigated.

2.2 Materials and Methods

2.2.1 Bacterial strains

The experiments were performed using *Pseudomonas putida* LV 2-4 and *Campylobacter jejuni*16-2R as model organisms. *P. putida* LV 2-4 (obtained from the Microbiology culture collection at Lakehead University) was cultured in Tryptic Soy Broth (TSB, Difco) at 37°C, overnight. *C. jejuni* 16-2R (donated by Dr. J. Odumeru, University of Geulph) was cultured in TSB supplemented with Campylobacter growth supplement (SR 084, Oxoid) under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) at 42°C for 48h.

2.2.2 Preparation of gradient and Buoyant Density Centrifugation

The gradient for BDC was formed according to Lindqvist (1995). Sterile Standard Isotonic Medium (SIM) consisting of 850 mg NaCl and 100 mg peptone in 100 ml of sterile Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) was diluted to 40% with sterile peptone water (8.5 g NaCl and 1.0 g peptone in 1L distilled water).

In a 1.5 ml Eppendorf tube, 0.9 ml of sample was carefully layered over 0.6 ml of the 40% SIM gradient and then centrifuged for 1 minute at 16,000 x g. After removing 1.4 ml supernatant, the remaining pellet was washed with 1.0 ml of sterile PBS, centrifuged at 9,500 x g for 5 minutes and 0.9ml supernatant was removed. The remaining volume (approximately 0.1 ml) was used to determine cell recovery.

2.2.3 Determination of cell recovery

The grown cell cultures were serially diluted in sterile Phosphate Buffered Saline (PBS, 0.14M NaCl, 2.68 mM KCl, 0.01 M Na_2HPO_4, 1.76 mM KH_2PO_4 at pH 7.4). Dilutions of 10^{-3}, 10^{-5}, and 10^{-7} were used for *P. putida* LV 2-4 and dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were used for *C. jejuni* 16-2R. For each dilution of *C. jejuni* 16-2R and *P. putida* LV 2-4, triplicate samples were tested. Cell numbers in the initial sample and in the final volume after the BDC procedure were determined by spiral plating. *P. putida* LV 2-4 was plated on Tryptic Soy Agar (TSA, Difco) and incubated at 37°C overnight. *C. jejuni* was plated on Tryptic Soy Blood agar (Difco) supplemented with 5% sheep blood and incubated for 48h at 42°C, microaerophilically.

2.2.4 Effect of temperature

The effect of temperature was evaluated by comparing the cell recovery of the BDC procedure when performed at either 4°C or 23°C. Dilutions of *P. putida* LV 2-4 were subjected to BDC in a thermal regulated centrifuge set at either 4°C or 23°C. The cell numbers in the initial and final samples were determined by spiral plating. Samples were plated on TSA and incubated at 37°C overnight.

2.2.5 Effect of enrichment medium containing blood

Broth cultures of *C. jejuni* 16-2R were serially diluted in Bolton Broth (Oxoid) supplemented with 5% sheep blood and dilutions of 10^{-1}, 10^{-3}, and 10^{-5} were subjected to the BDC procedure at 4°C. Cell numbers in the initial and final sample were determined by spiral plating. Samples were plated on Tryptic Soy Blood Agar supplemented with 5% sheep blood and incubated at 42°C for 48h, microaerophilically.

2.2.6 Effect of chicken homogenate on BDC

Chicken homogenate was prepared by weighing 10g of raw chicken pieces into 90 ml of sterile peptone water and pulsifying the mixture in a sterile stomacher bag (Fisher Scientific) for 2 minutes. Serially diluted *C. jejuni* culture was added to the chicken homogenate and the mixture was processed with BDC. For comparison, non-treated control samples were prepared by centrifuging 0.9ml of each dilution for 10 minutes at 21,000 x g and removing 0.8ml of supernatant. All samples were subsequently stained prior to confocal microscopy. For each sample 100µl of 0.5 µM Sytox Green (Molecular Probes, Oregon) was added. After standing for 5 minutes at room temperature in the dark, the mixture was filtered through a 0.22µm black

polycarbonate membrane filter (GTBP 02500, Millipore, Billerica, Ma) and then washed with 10 ml of PBS. Filters were transferred to glass slides and allowed to air dry in the dark at room temperature. Subsequently, 15µl of antifade mounting oil (Molecular Probes, Oregon) was placed on the filters prior to addition of a coverslip. Samples were then examined using confocal microscopy (Olympus FV300, Olympus America). Excitation was with an argon laser at 488nm. Emission was detected through a band pass filter with 510nm-530nm.

2.3 Results

2.3.1 Evaluation of temperature effect on BDC

Since the BDC protocol results in a sample concentration (900µl to 100µl), comparisons were made for total cell numbers. No differences were found between BDC performed at the two temperatures (Figure 2.1). A comparison of recovery at different cells levels revealed some variations at 23°C, where cell loss was observed at high cell counts and an increase was seen at low cell counts (Figure 2.2B). No such variations were observed at 4°C (Figure 2.2A). Based on these results, all subsequent BDC treatments were performed at 4°C.

2.3.2 Cell recovery of BDC

Overall, there was no difference in cell numbers before and after BDC for both *C. jejuni* and *P. putida* (Figure 2.4 and 2.6) and the values before and after BDC treatment were linearly correlated (Figures 2.3 and 2.5). The BDC procedure results in a concentration of cells because the sample volume is reduced from 900µl to 100µl. An increase in cell concentrations was seen for both *P. putida* LV 2-4 and *C. jejuni* 16-2R (data not shown).

2.3.3 Effect of enrichment medium with blood

The presence of blood in an enrichment medium clearly affected cell recovery. For all levels of cell counts examined, cell numbers were lower after the BDC procedure (Figure 2.7 and 2.8). However, though, there was an average loss of 0.5-0.8 $\log_{10}$, there was a considerable increase in cell concentration of about 0.5 $\log_{10}$ CFU/ml.

2.3.4 Effect of BDC on removal of chicken homogenate

For some samples not treated with BDC, the debris of chicken meat was so large that the coverslip could not be placed. Samples treated with BDC showed a substantial decrease in the size and number of particles present when compared to non-treated samples (Figure 2.9).

2.4 Discussion

Previous studies have suggested that BDC is an ideal preparation step for samples prior to testing by PCR. These conclusions were based on improvements in the detection limit of the particular protocol being examined. Although this information is important to determine whether the procedure is capable of removing inhibitory particles, it does not evaluate the efficiency of the BDC procedure in terms of cell recovery and cell concentration. Only few experimental data have been published comparing cell counts before and after the BDC procedure, and it is therefore difficult to compare the results of this study to those of previous studies.

The effect of temperature on cell recovery was examined to ensure that performing the BDC at either refrigerated or room temperature would not influence the effectiveness of the density gradient. The comparison of the two temperatures showed no significant difference,

suggesting the BDC procedure was not affected by temperature (Figure 2.1). Although, no overall loss or gain of cells was found for both temperatures, the BDC procedure performed at 23°C had significant differences at selected ranges of cell counts. At low cell counts, a loss of cells was observed, while significant gains were found for higher cell counts (Figure 2.2). Since no variation in cell recovery was observed at 4°C, it is suggested to perform the procedure at 4°C. The performance of Percoll at different temperatures was also demonstrated by Woldringh et al (1981), who examined changes in the buoyant density of *E. coli* cells using varying concentrations of Percoll. Considerably narrower bands of cells formed when the Percoll solution and centrifuge were kept at refrigerated temperatures. At temperatures above 20°C, the bands were wider, suggesting that the separation of particles was not as successful as when the Percoll was kept cold. The authors suggested that this could be due to the increased density of Percoll and cells at lower temperatures (Woldringh et al, 1981).

The efficiency of the BDC procedure was evaluated by comparing cell numbers before and after treatment. Due to the potentially low numbers of *C. jejuni* present in poultry samples, it is important to avoid any cell loss, if the BDC method is used as a means of removing inhibitory particles prior to FISH testing. Overall, the BDC procedure did not result in any loss of cells at low levels for both *C. jejuni* 16-2R and *P. putida* LV 2-4. At both high cell counts of *P. putida* LV 2-4 and medium cell counts of *C. jejuni* 16-2R there appeared to be slight loss of cells, although statistically they were not different. A study by Uyttendaele et al (1999), found a similar loss of 0.1-0.8 log units when cells counts were in the range of 10^3-10^5 CFU/ml. The authors suggested that this apparent loss of cells is acceptable, as no food microbiology procedure is guaranteed to have a 100% cell recovery. A study by Halldórsdóttir et al (2002) used BDC to separate *Mycobacterium paratuberculosis* prior to detection with PCR. By

examining the results of PCR with and without the application of BDC, a theoretical CFU limit to the BDC procedure was determined. The authors suggested that 1000 CFU/g was the lowest concentration of cells that could be recovered accurately. Although at lower counts of 1-10 CFU some samples appeared positive, the results were inconsistent, suggesting that at low cell counts the procedure may not always be capable of recovering the target cells.

The current study showed some loss of cells, but this is offset by significant increases in sample concentration. For both high cell counts of *P. putida* LV 2-4 and all cell counts of *C. jejuni* 16-2R, an average of 0.6-1.5 $\log_{10}$ increase in cell concentration was observed. Uyttendaele et al (1999) showed a similar increase in cell concentration for two strains of *C. jejuni*. This concentration effect is due to the sample volume being reduced from 900µl to 100µl, an almost 10 fold increase in concentration.

Due to the low numbers of *C. jejuni* normally present in poultry, enrichment of the sample may be required prior to testing with FISH. Bolton Broth supplemented with 5% blood, a commonly used medium in the poultry industry (Hunt et al, 1998), was spiked with *C. jejuni* and treated with BDC to ensure that the blood would not affect the cell recovery. However, a loss of cells was observed at all cell counts. Since only minor cell loss was observed with samples diluted in buffer, it is clear that the blood reduces the efficiency of the BDC procedure. This may be due to either the blood retarding the *C. jejuni* from completely moving through the gradient or to clumping of the bacteria, therefore reducing the number of colony forming units. Because the BDC procedure concentrates cells, an average 0.5 $\log_{10}$ increase in cell concentration was observed despite the cell loss.

Initial experiments to determine cell recovery and cell concentration were performed using cultures grown in nutrient medium, free of food particles. In naturally contaminated

samples *C. jejuni* would be in a homogenate of poultry meat, liquid from carcass washes or water collected from processing. Therefore, the ability of BDC to remove particulate matter from a chicken homogenate was evaluated microscopically. The size and number of particles present before and after the samples were run through the density gradient were determined. Chicken homogenate samples not treated with BDC were often difficult to examine because large particles interfered with placing the coverslip and focusing the microscope. In contrast, samples treated with BDC were easier to handle because the sample pellet formed after the procedure was considerably smaller than that of non-treated samples. A considerable decrease in the number of chicken particles was seen and those particles still present were also significantly smaller. The presence of chicken particles after BDC indicates that the buoyant density of those particles is similar to that of *Campylobacter* which enabled them to move through the gradient.

The current study demonstrated the ability of BDC to not only remove inhibitory particles interfering with FISH, but to also concentrate cells, which may contribute to lowering the detection limit of the FISH procedure.

Figure 2.1 Cell recovery of BDC performed at 4°C and 23°C using *P. putida* LV 2-4.

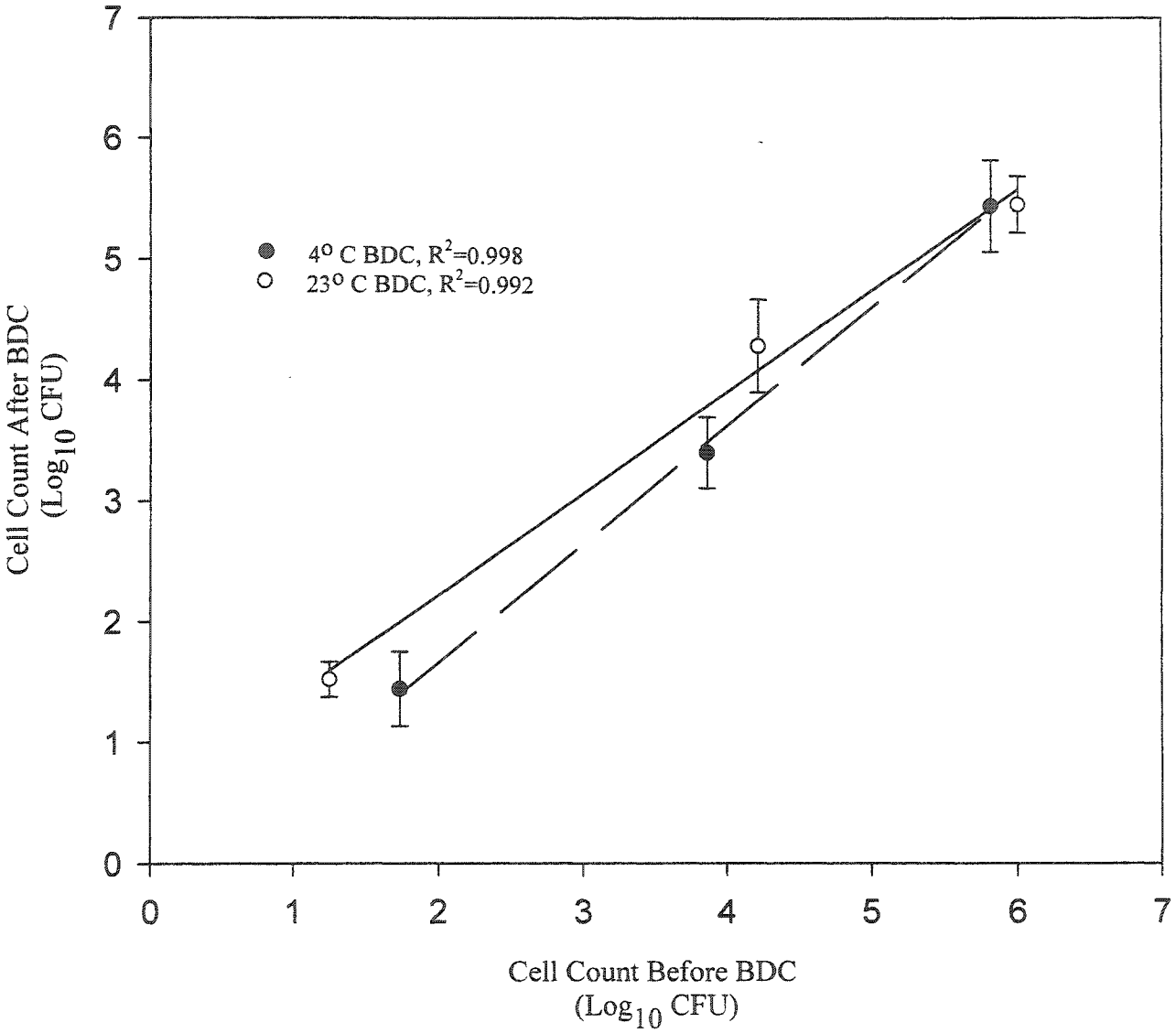
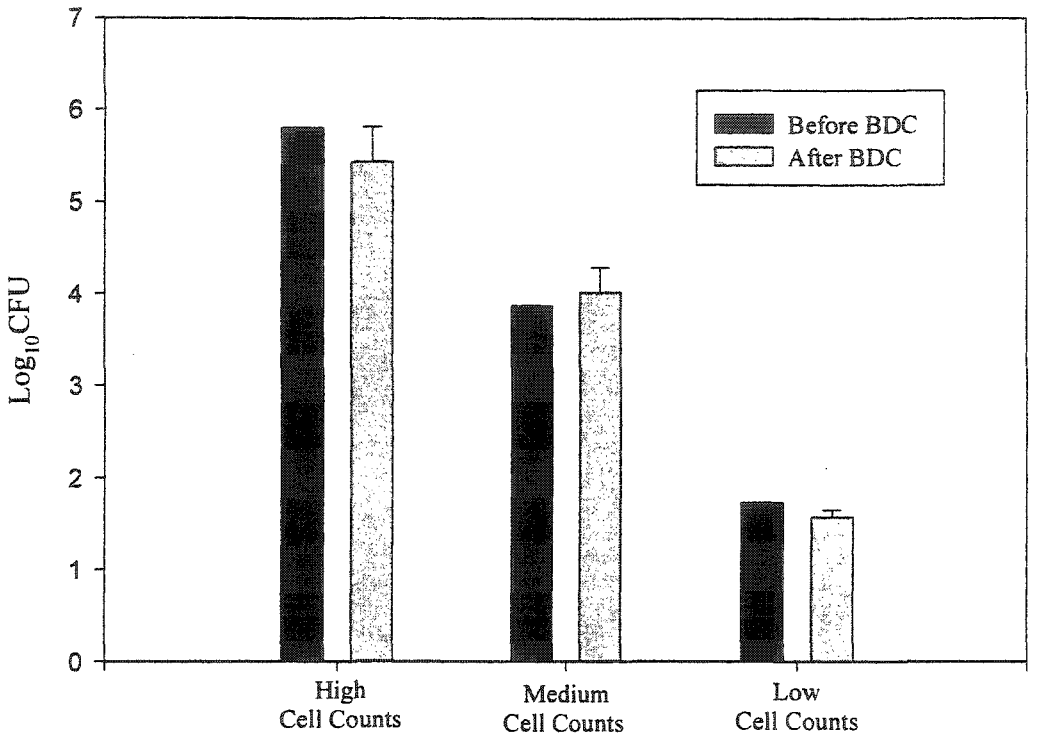


Figure 2.2 Cell counts before and after BDC performed at 4°C and 23°C.

A. BDC at 4°C



B. BDC at 23°C

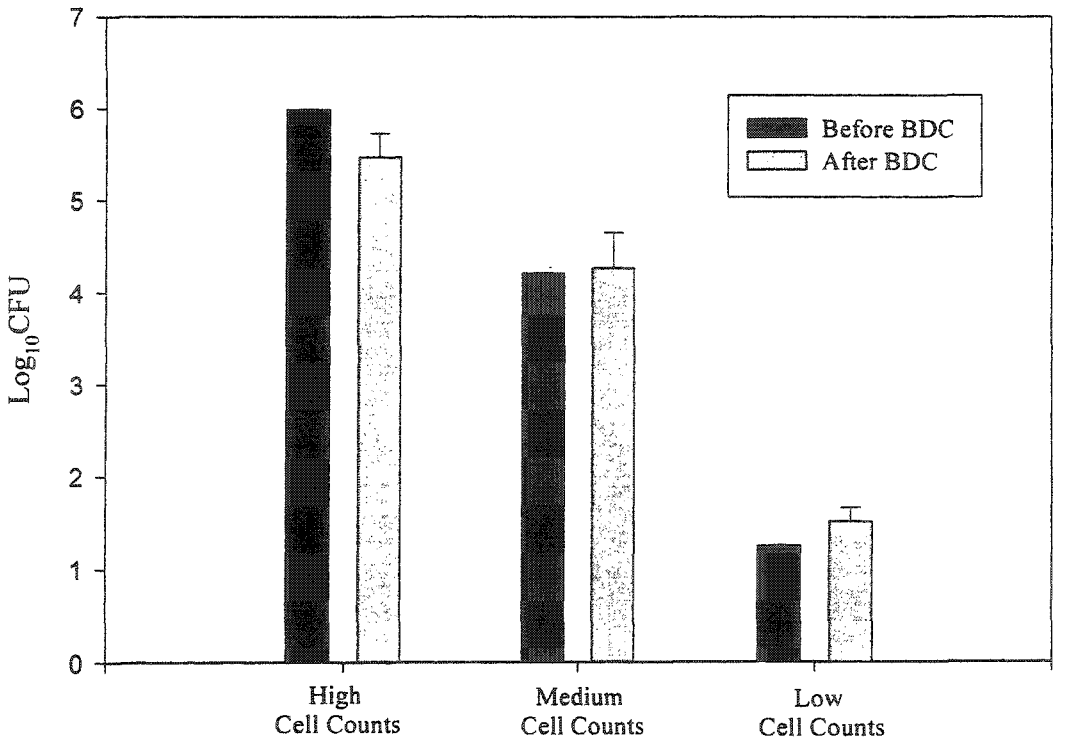


Figure 2.3. Linear correlation of cell counts before and after BDC for *C. jejuni* 16-2R.

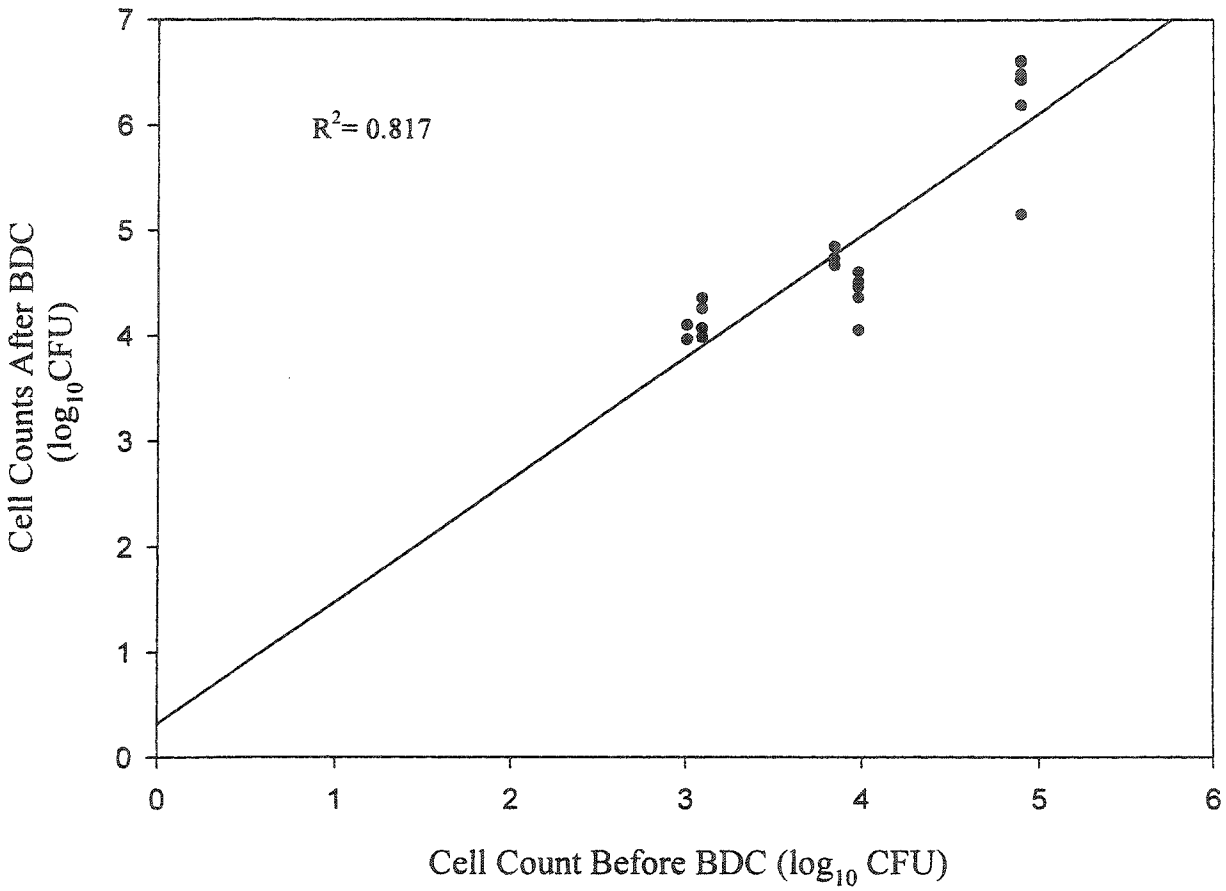


Figure 2.4 Cell counts before and after BDC for *C. jejuni* 16-2R.

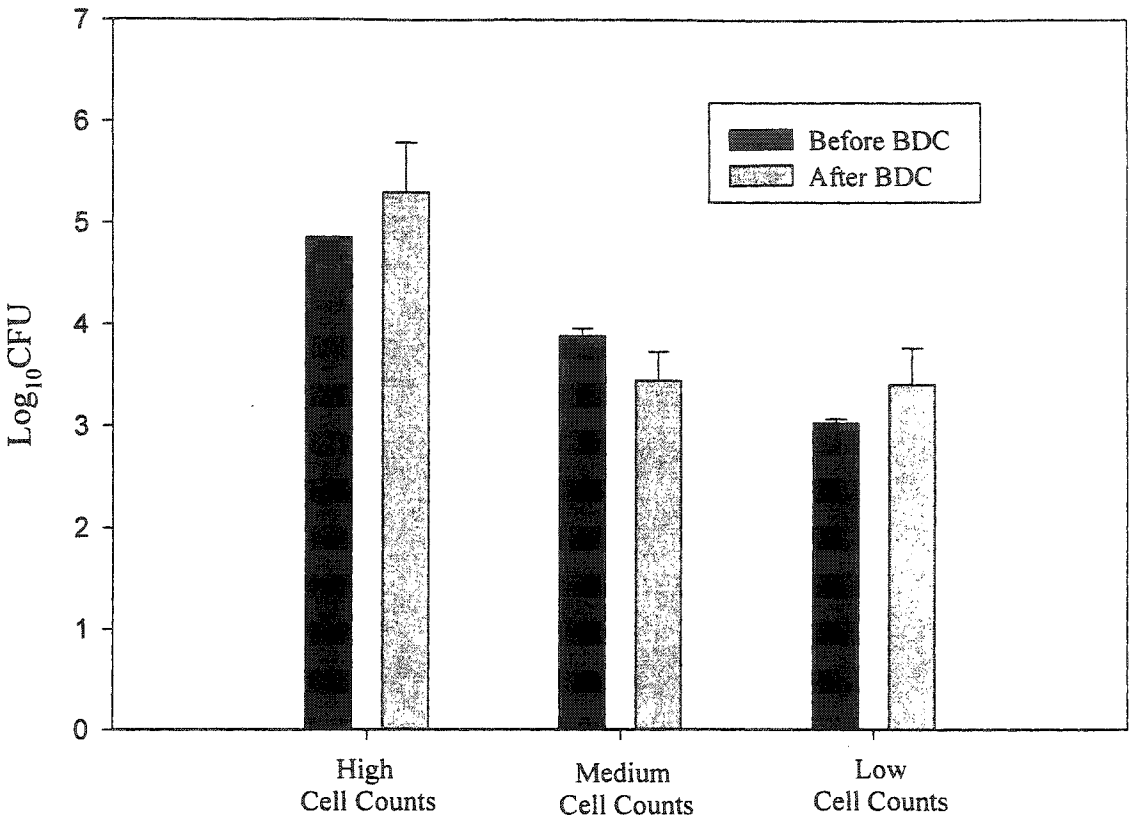


Figure 2.5 Linear correlation of cell counts before and after BDC for *P. putida* LV 2-4.

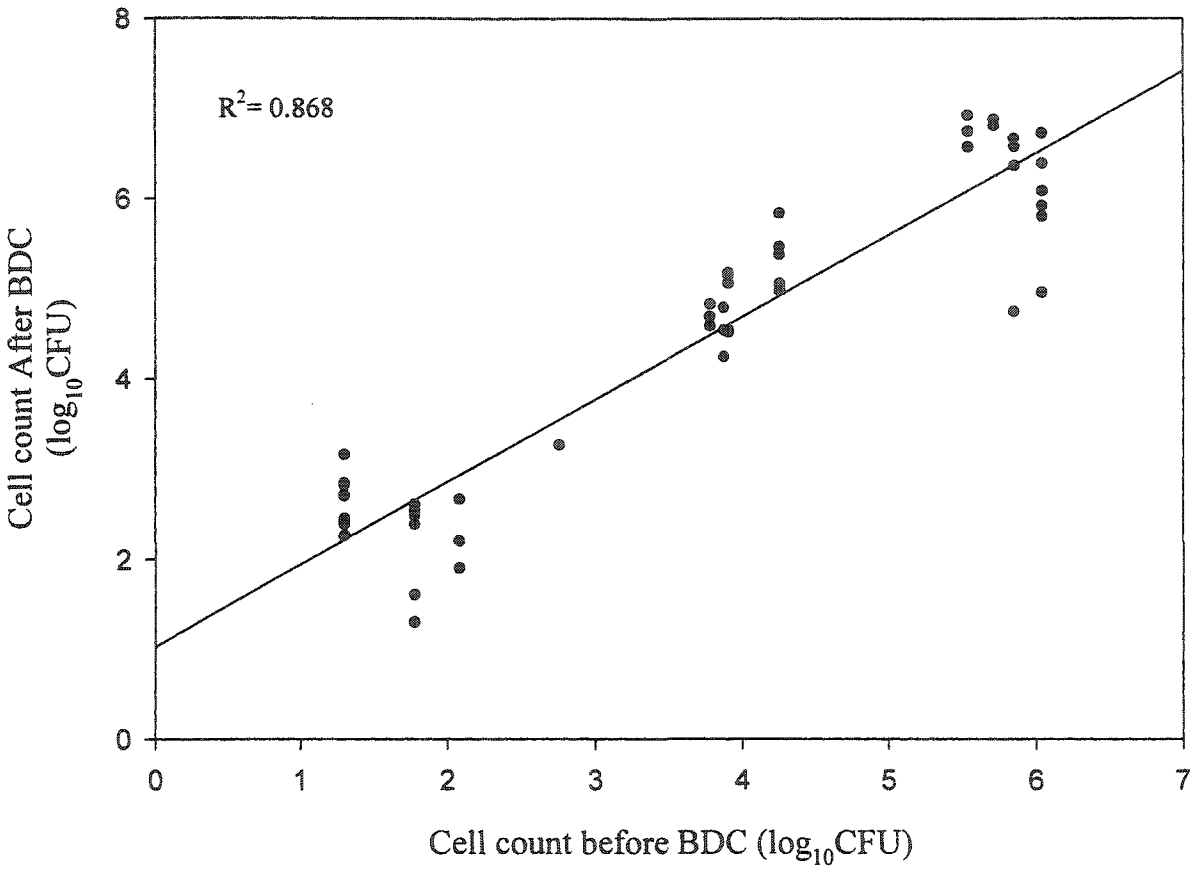


Figure 2.6 Cell counts before and after BDC for *P. putida* LV 2-4.

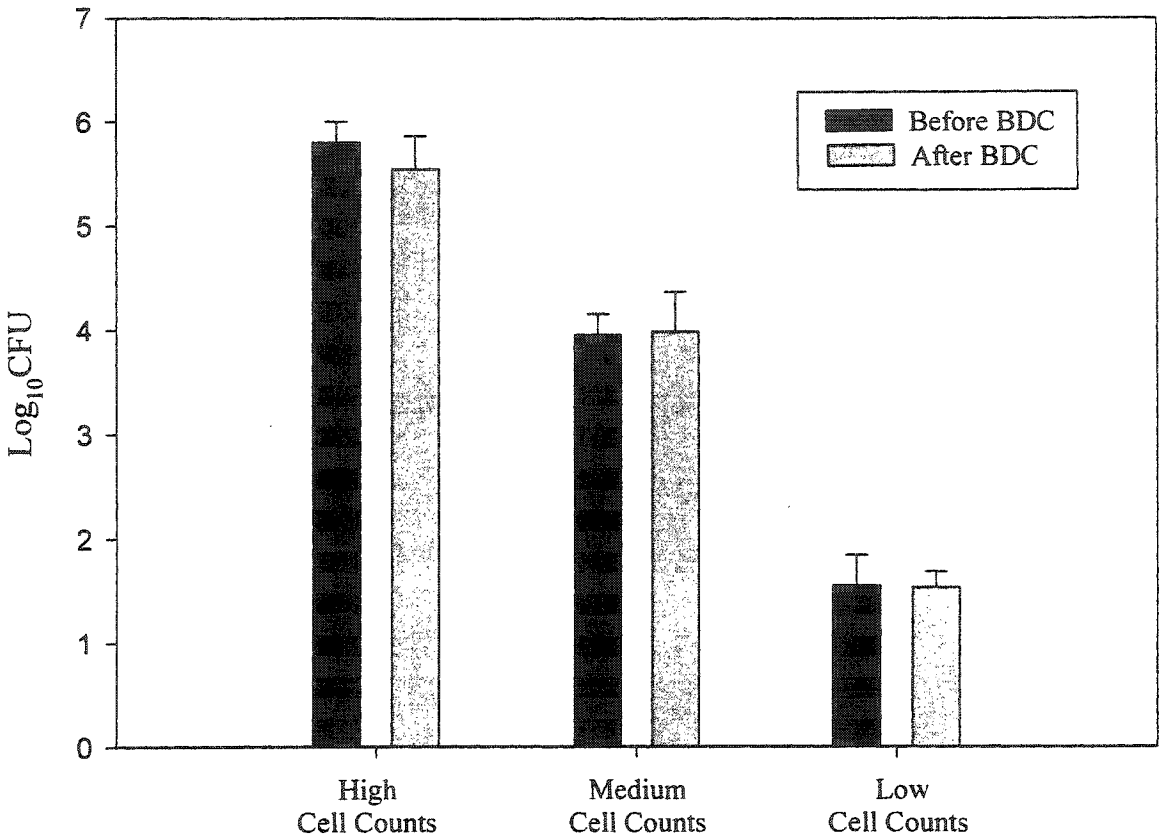


Figure 2.7 Cell counts before and after for *C. jejuni* 16-2R in Bolton Broth with blood.

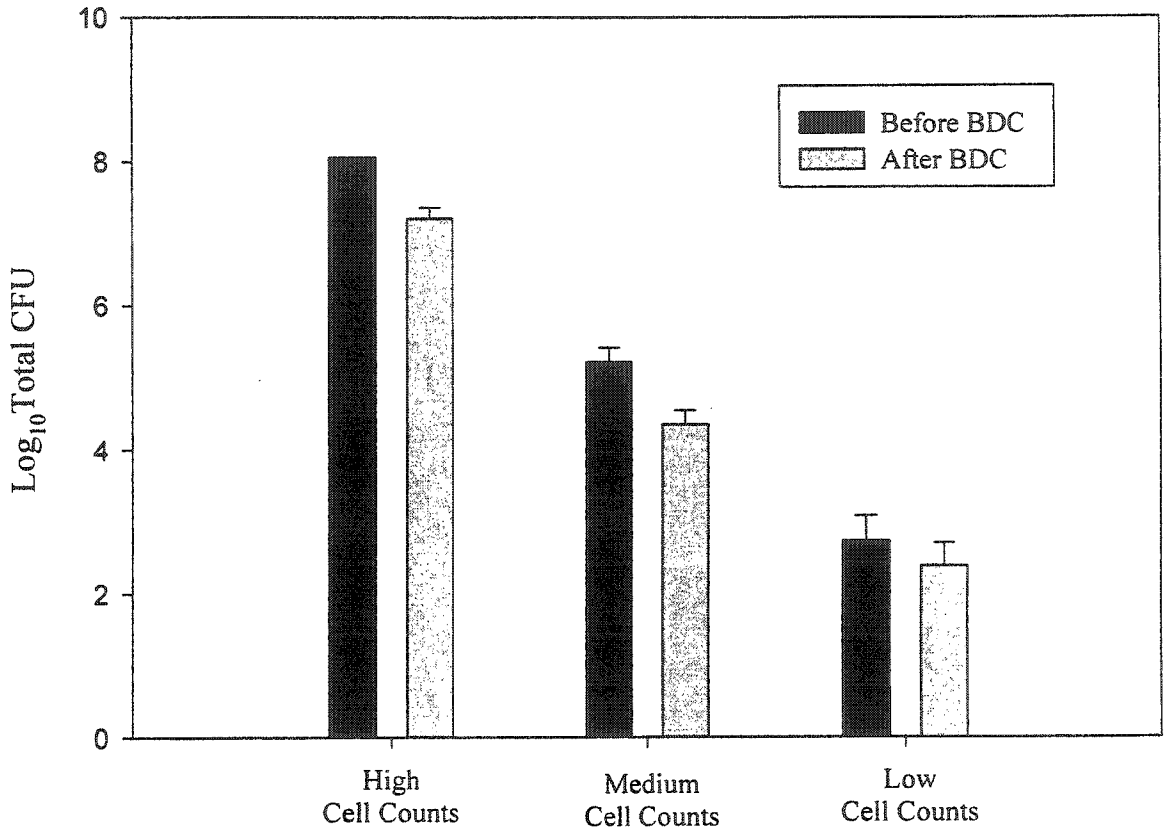


Figure 2.8 Cell counts before and after BDC with Bolton Broth with blood supplement.

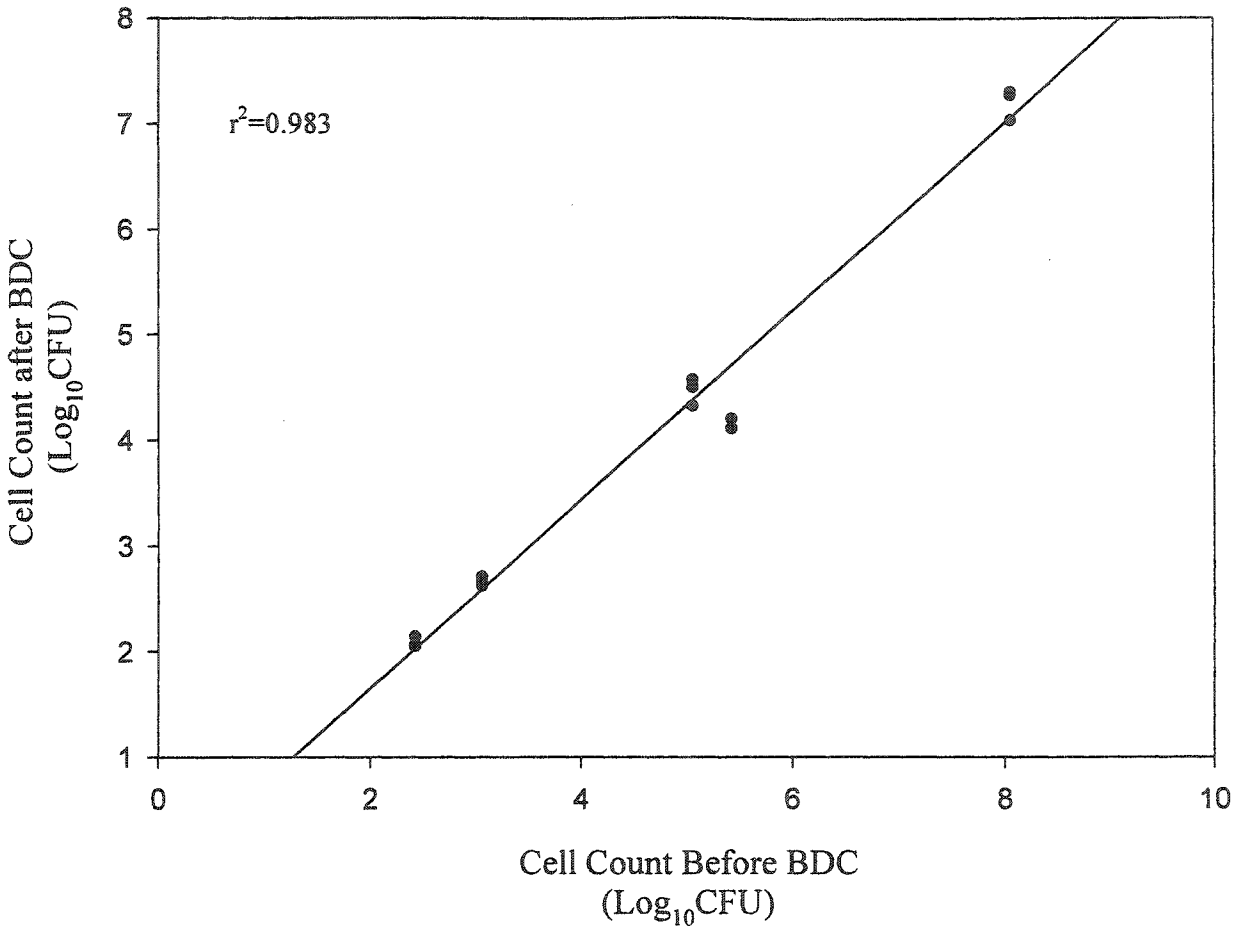
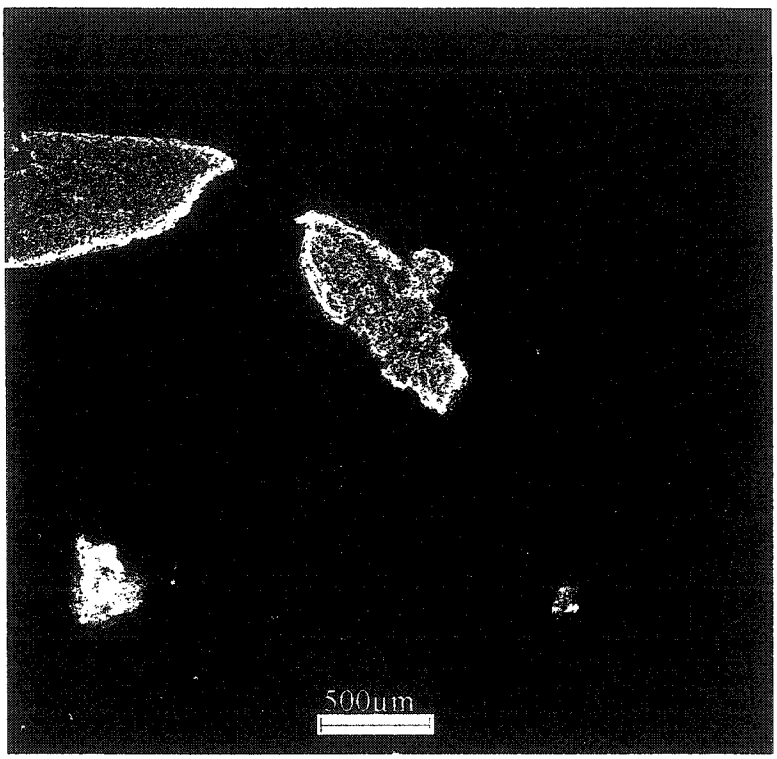
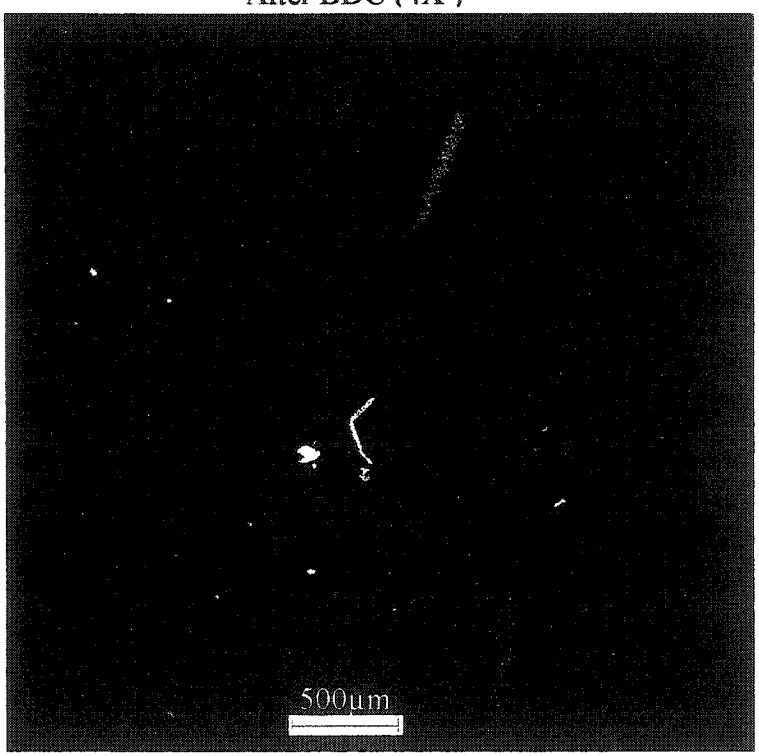


Figure 2.10 Confocal microscopy images of chicken homogenate samples before and after BDC.

Before BDC (4X)



After BDC (4X)



3. Evaluation of Fluorescent *in situ* hybridization for the detection of *Campylobacter jejuni*

3.1 Introduction

Fluorescent *in situ* hybridization (FISH), as described in Chapter 1, is a molecular-based detection method, which uses fluorescently-labelled rRNA probes to target specific microorganisms. Due to the specificity of the rRNA sequence used to design the probe, target organisms can be detected in samples containing a variety of microbes. Traditionally, this procedure has been used to examine microbial populations in ecological systems, but it has recently been introduced into both food and clinical microbiology. In the quest for a molecular based, rapid and sensitive detection method for *Campylobacter* spp., FISH may prove to be a suitable alternative to the polymerase chain reaction (PCR), the molecular detection method most commonly evaluated for food microbiology applications. Moreno et al (2001) suggested that, unlike PCR, FISH is not affected by inhibitory substances frequently found in DNA extracts from food. Also, larger sample volumes can be processed and a positive signal observed directly in the sample by microscopy.

Since the FISH procedure is relatively new for the food industry, there exists only limited published information demonstrating detection of *Campylobacter* spp. Moreno et al. (2001) compared the ability of FISH and PCR to detect *Campylobacter* in poultry and reported that both techniques were suitable, but FISH was less sensitive than PCR. This lower sensitivity can be overcome by a brief enrichment step. A benefit of FISH is its lower rate of false-positives due to dead cells, because rRNA targeted by the test is degraded soon after cell death, while DNA can remain intact over long periods of time. This notion is supported by a study of Moreno et al. (2003), who used both FISH and PCR to detect *Campylobacter* and the closely related genus

Arcobacter in water and sewage samples. Eight of 40 samples were positive with PCR, but negative with the culture method. Only three of these culture negative samples were positive with FISH. The authors suggest that conventional plating, and possibly PCR, may be a poor means of detecting organisms present in stressful environments as VBNC cells are not detected. In contrast however, a total of 10 samples were found positive with the culture method and all of them had a positive PCR result, but three were negative with FISH. Overall, FISH may have good potential as a molecular based, rapid and sensitive detection method for *Campylobacter* spp. in food, but a more detailed evaluation of achievable detection limits seems warranted.

The objective of this chapter was therefore to develop and evaluate a FISH protocol for the detection of *C. jejuni* in poultry. One of the main goals was to ensure that any enrichment step required would allow aerobic incubation, preferably in a blood-free medium, reducing the cost for industry and the hindrance seen with the blood.

3.2 Materials and Methods

3.2.1 Bacterial strains

The 21 *C. jejuni* isolates and the 16 non-*Campylobacter* strains used in this study are listed in Tables 3.1 and 3.2. *C. jejuni* cultures were donated by Dr. J. Odumeru, Laboratory Services Division, University of Guelph and non-*Campylobacter* cultures were obtained from the Microbiology culture collection at Lakehead University. Each isolate, was grown at 42°C for 48h, microaerophilically (5% O_2, 10% CO_2 and 85% N_2) in Tryptic Soy Broth (TSB) supplemented with Campylobacter growth supplement (SR 084, Oxiod). All non-*Campylobacter* isolates, excluding *Clostridium perfringes* ATTC 13124, were grown in TSB at

37°C, overnight. *C. perfringes* ATTC 13124 was grown anaerobically in TSB at 37°C, overnight.

3.2.2 FISH reagents and buffers

3.2.2.1 Probe

An oligonucleotide with the sequence of 5' GTGTTAAGCAGGAGTATAGAG 3', fluorescently labelled with 6-TAMRA at the 5' end, was used for the detection of *Campylobacter* by FISH. The probe had been prepared by SIGMA Genosys (Oakville, Ontario). It was kept as a stock solution of 0.1nmol/µl in water at -30°C.

3.2.2.1 DEPC water

Diethylpryocarbonate (DEPC) is a chemical that inhibits RNase activity and all solutions used in the FISH procedure were prepared with 2% DEPC treated water (DEPC water). Two ml of DEPC was added to 1L of ultrapure water, stirred constantly overnight and then autoclaved at 121°C for 20 minutes.

3.2.2.2 Hybridization buffer and wash buffer

Hybridization buffer consisted of 20 mM Tris-HCl (pH 8.0), 0.1% sodium dodecyl sulfate (SDS), 0.01% yeast tRNA and 1.0 X saline sodium-citrate (SSC). A 1.0 X SSC solution consists of 0.15 M NaCl and 0.015 M sodium citrate. The solution was sterilized by autoclaving at 121°C for 20 minutes.

Wash buffer was used at 1 X SSC and 0.1 X SSC. These solutions consisted of 20 mM Tris-HCl and 0.1% SDS in 1 X and 0.1 X SSC, respectively.

3.2.3 FISH procedure

3.2.3.1 Fixation and permeabilization of cells

Prior to FISH, bacterial cells were pelleted by centrifugation, resuspended in 1ml of 4% paraformaldehyde (Fisher Scientific) and left to stand at room temperature for 30 minutes. The dead bacteria were pelleted by centrifugation for 10 minutes at 9,500 x g. Supernatant was removed and the bacterial cells were permeablized in a series of ethanol wash steps with concentrations of 50%, 75%, 95% and 100% ethanol/water (v/v%). For each step, 1ml of ethanol solution was added to the sample and vortexed. The cells remained in solution for 2 minutes, followed by centrifugation for 10 minutes at 21,000 x g.

3.2.3.3 Hybridization

Prior to the addition of the probe (0.7pmol/μl of hybridization buffer), the hybridization buffer was heated in a 70°C incubator for 15 minutes. Each sample was suspended in 50 μl of hybridization buffer with probe and then incubated for 1h at 70°C.

To remove unbound or non-specifically bound probe, samples were washed 3x in 1 X SSC wash buffer and 3x in 0.1 X SSC wash buffer. For each wash step, the sample was suspended in 1ml of wash buffer and incubated for 10 minutes at 70°C. The sample was then centrifuged for 10 minutes at 9,500 x g and 1ml of supernatant was removed. After the last wash step, the sample was resuspended in 1ml of DEPC water and centrifuged for 10 minutes at 9,500 x g. A 900μl volume of supernatant was removed and the remaining 100μl sample was examined using either confocal microscopy or a fluorescence plate reader.

3.2.4.2 Examining samples with confocal microscopy

To visualize all cells present, each 100µl sample was stained in 100 µl of 5µg/ml 4',6-diamidino-2-phenylindole (DAPI) with a 5 minute exposure at room temperature in the dark. The sample was then filtered onto a 0.22µm black polycarbonate membrane filter (GTBP 02500, Millipore, Billerica, Ma) and rinsed once with 1ml PBS. The filters were transferred to glass slides and allowed to air dry in the dark. A drop of anti-fade reagent in glycerol buffer (Molecular Probes, Oregon) was placed between the filter and the coverslip. When necessary, the slides were stored up to 1 day at -30°C before performing microscopy. Microscopy was performed with an Olympus BX51 Epifluorescence microscope. The DAPI signal (total cells) was examined using an excitation wavelength of 450-480nm and an emission filter of 515nm. The FISH signal (*Campylobacter* cells) was seen using an excitation of 530-550nm and an emission filter of 590nm.

3.2.3.4 Examining samples using a fluorescence plate reader

Each 100µl sample was placed in a well of a 96 well black assay plate (Costar 3916, Corning Incorporated, NY) and then read in a fluorescence plate reader (FLUOstar Optima, BMG LABTECH, Germany) at gain 3016, using a 544nm excitation filter and a 595nm emission filter. Each plate was read five consecutive times at identical settings and the results were used to calculate average fluorescence with standard deviation.

3.2.4 Specificity and cross-reactivity of FISH assay

Specificity of the probe was tested with 21 *C. jejuni* isolates mixed with a *P. putida* LV 2-4 culture (specificity) and 16 non-*Campylobacter* isolates mixed with a culture of *C. jejuni* 16-

2R (cross-reactivity). For each test, 500µl of a grown *C. jejuni* culture (approx. 10^8 CFU/ml) was mixed with 500µl of the grown non-*Campylobacter* culture (diluted to 10^5 - 10^6 CFU/ml) and then centrifuged for 10 minutes at 21,000 x g. The samples were resuspended in 1ml of 4% paraformaldehyde, treated with the FISH protocol and examined using confocal microscopy (Section 3.2.3).

Probe specificity was determined by observing the number and size of the cells stained with DAPI and comparing to cells with a positive FISH signal. Only *C. jejuni* was to show a FISH signal, but not *P. putida* LV 2-4 or any other non-*Campylobacter* isolate, which should only fluoresce with DAPI. A probe signal from a non-*Campylobacter* isolate would represent non-specific binding.

3.2.5 Probe detection limits

To determine the detection limit and limit of quantitation of unhybridized probe, the probe was serially diluted in sterile hybridization buffer for concentrations ranging from 7.0 x 10^2 pmol/ml to 0.7 x 10^{-2} pmol/ml. For each dilution, 90µl were added into the wells of a microtitre plate followed by examination with the fluorescence plate reader as described in Section 3.2.3.4. This analysis was replicated 3 times with independently prepared probe dilutions.

To determine the detection limit and limit of quantitation for *C. jejuni* cells, 10ml of a 48h culture of *C. jejuni* 16-2R was centrifuged for 10 minutes at 4,000 x g and the pellet resuspended in 1.5 ml of sterile PBS. Serial dilutions were prepared in PBS and 1ml samples ranging from 7.9 x 10^9 to 3.6 x 10^3 CFU/ml were centrifuged for 10 minutes at 21,000 x g. The

supernatant was removed and the pellets resuspended in 4% paraformaldehyde, followed by the FISH procedure and analysis with the fluorescence plate reader (Section 3.2.3).

3.2.6 Evaluation of enrichment media

Growth of *C. jejuni* over 48h was evaluated in four different media types which were incubated microaerophilic and aerobically; namely TSB with Campylobacter Growth supplement (TSB-Sup), Bolton Broth with Campylobacter Growth supplement (BB-Sup), Bolton Broth with 5% sheep blood (BB-blood) and a semi-solid aerobic medium (Jeffery et al, 2000). In addition, the effect of the natural flora present in chicken meat on the growth of *C. jejuni* in BB-blood was evaluated.

3.2.6.1 Preparation of media

For both TSB-Sup and BB-Sup (CM 0983, Oxoid), 500ml of each medium were prepared according to the manufacturer's instructions and autoclaved for 20 minutes at 121°C. After cooling to room temperature, 1 vial of Campylobacter Growth supplement (SR 084, Oxoid) was added to each medium.

The semi-solid aerobic medium was prepared according to Jeffery et al (2000), excluding the addition of antibiotics.

BB-blood was prepared by autoclaving the Bolton Broth for 20 minutes at 121°C and then cooling to room temperature, prior to adding sheep blood.

For samples spiked with chicken meat, Bolton Broth Selective Supplement (SR 183, Oxoid) was added to 500ml volumes of sterile BB-blood. The medium was then spiked with

chicken meat as follows: 25g of chicken meat were placed into 225ml of sterile broth and pulsified for 2 minutes in a net lined stomacher bag.

3.2.6.2 Comparison of growth of C. jejuni

For both TSB-Sup and BB-Sup, 100ml of medium was pipetted into a sterile 250ml flask and covered with aluminium foil. For BB-blood samples (sterile and spiked with chicken), the medium was pipetted into a sterile 250ml screw-top flask, which was filled to within 20mm of the top (Oxoid, Ltd.).

A 48h culture of *C. jejuni* 16-2R was diluted in PBS to 10^{-5} and 100µl of the dilution added to each broth, mixed and incubated at 42°C for 48h, microaerophilically and aerobically. The semi-solid medium was only incubated aerobically.

At 24 and 48h *C. jejuni* counts were determined by spiral plating onto Tryptic Soy Blood Agar with 5% sheep blood, and incubated microaerophilically at 42°C for 48h. Each broth/ atmosphere combination was tested in three independent replicates.

3.2.7 Fluorescence of sample blanks

Five different *Campylobacter*-free samples (1ml) were treated with FISH to determine the average background fluorescence. They included DEPC water (Section 3.2.2), Tryptic Soy Broth spiked with *P. putida* LV 2-4 at 4.2 x 10^8 CFU/ml, BB-blood (Section 3.2.6.1), BB-blood spiked with chicken meat (Section 3.2.6.1) and BB-blood after lysis treatment. The three samples with BB-blood were incubated microaerophilically at 42°C for 48h before analysis of the blank signal. To lyse BB-blood, 1ml of sterile ultrapure water was added to 1ml of medium and vortexed. After 2 minutes at room temperature, the samples were centrifuged at 3,200 x g

for 10 minutes. For the other sample types, 1ml was centrifuged at 21,000 x g for 10 minutes. Supernatants were removed, the pellet resuspended in 4% paraformaldehyde, followed by treatment with FISH and examination by a fluorescence plate reader (Sections 3.2.2 and 3.2.3).

3.2.8 Removal of blood and background matter prior to FISH

Preliminary experiments with naturally contaminated samples, combining enrichment in BB-blood and FISH, showed that incubation altered the medium resulting in significant increase of sample debris which interfered with FISH. Thus, four approaches to reduce sample debris were evaluated: i) lysing the blood, ii) lysing the blood and treating with BDC, iii) lysing the blood and filtering through a 0.8μm membrane filter, and iv) combination of lysing, BDC and filtering.

3.2.8.1 Sample type

Each treatment was tested with and without a 48h incubation at 42°C (microaerophilic) and with and without *C. jejuni* present. BB-blood spiked with chicken meat was used for all trials, prepared as described in Section 3.2.6.1. Both 1ml and 10ml volumes were tested with one replicate read five times with the fluorescence plate reader.

Non-incubated samples were either used directly (blank) or spiked with 10^8 CFU/ml of *C. jejuni* 16-2R. The *C. jejuni* cells used for spiking had been grown as described in Section 3.2.1, pelleted by centrifugation and the pellets were suspended in the appropriate volume of broth. Samples were then analysed without further treatment.

For incubated samples, the broth was either used directly (blank) or inoculated with 10 CFU of *C. jejuni* 16-2R (sample). Both blanks and inoculated samples were then incubated for

48h at 42°C, microaerophilically. Prior to FISH testing, *C. jejuni* counts were determined by spiral plating onto Tryptic Soy Blood Agar with 5% sheep blood, incubated microaerophilically at 42°C for 48h. Samples inoculated with *C. jejuni* inoculated samples reached 4.8 x 10^7 CFU/ml after enrichment and no *C. jejuni* were present in enriched blanks.

3.2.8.2 Lysing of blood

All samples were treated to lyse the blood in the medium. For both 1ml and 10ml volumes, an equal volume of sterile ultrapure water was added to the sample and vortexed. The sample was then allowed to stand for 2 minutes, followed by centrifugation at 3,200 x g for 10 minutes and removal of supernatant. For samples treated with lysing only, the pellet was resuspended in 1ml of 4% paraformaldehyde followed by FISH treatment and examination in the fluorescence plate reader (Sections 3.2.2 and 3.2.3). Otherwise, samples were processed by one of the protocols described in Sections 3.2.8.3, 3.2.8.4 or 3.2.8.5.

3.2.8.3 Lysing of blood and filtering through a 0.8μm membrane filter

The pellet formed after lysis treatment was resuspended in 10ml of sterile PBS and passed through a sterile 0.8μm polycarbonate membrane filter (ATTP02500, Fisher Scientific) The filtrate was centrifuged at 3,200 x g for 10 minutes, the supernatant removed and the pellet was resuspended in 1ml of 4% paraformaldehyde, treated with FISH and examined by fluorescence plate reading (Sections 3.2.2 and 3.2.3).

3.2.8.4 Lysing and BDC

The pellet formed after the lysis treatment was resuspended in 1ml of sterile PBS and treated with the BDC procedure as described in Section 2.2.2. Samples were then resuspended in 4% paraformaldehyde, followed by treatment with FISH and examination in the fluorescence plate reader (Sections 3.2.2 and 3.2.3).

3.2.8.5 Lysing, BDC and filtering

Samples were treated as described in Section 3.2.8.4, but prior to suspension in 4% paraformaldehyde, samples were resuspended in 10ml of sterile PBS followed by filtration through sterile 0.8μm membrane filter and subsequent analyses as described in Section 3.2.8.3.

3.2.8.6 Lysing, BDC and filtering with application of prefilters

Samples were treated as described in Section 3.2.8.5, with a modification of the filtration step. One of four prefilters was mounted above the 0.8μm filter membrane in order to reduce clogging. The prefilters used were APFA02500 (1.6μm retention) and APFB02500 (1.0μm retention) both of which were borosilicate microfibre glass without binders, and AP2002500 (1.0μm retention) and AP2502500 (1.0μm retention) which were both borosilicate microfibre glass with acrylic binder resin (Fisher Scientific).

3.2.8.7 Effects of filtration on cell counts

Effects of filtration on *C. jejuni* counts were determined by plate counting. *C. jejuni* were enumerated before and after each treatment by spiral plating onto Tryptic Soy Blood agar with 5% blood which was incubated microaerophilically for 48h at 42°C. Both APFB02500 and

APFA02500 were tested in 6 replicates, while prefilters AP2502500 and AP2002500 were performed in triplicate.

3.3 Results

3.3.1 Probe specificity

Confocal microscopy demonstrated that the probe, was specific for the 21 isolates of *C. jejuni* tested (Table 3.1). FISH performed on non-*Campylobacter* isolates showed no apparent non-specific probe hybridization (Table 3.2).

3.3.2 Detection of probe and C. jejuni

For both unhybridized probe and *C. jejuni* hybridized with probe, the detection limit and limit of quantitation were determined using equations 3.1 and 3.2 (Harris, 2002).

Equation 3.1 Detection Limit = $Blank_{Ave} + 3 \times Blank_{STDEV}$

Equation 3.2 Limit of Quantitation = $Blank_{Ave} + 10 \times Blank_{STDEV}$

$Blank_{Ave}$ = Average of blank readings

$Blank_{STDEV}$ = Standard deviation of blank readings

The detection limit (Equation 3.1) represents those samples with fluorescence above 99% of the blank readings (Harris, 2002). This can also be used to determine the concentration of sample required for fluorescence above the detection limit. The limit of quantitation (Equation 3.2) is the level of fluorescence which can be used to quantitatively correlate the amount of fluorescence to the concentration of sample (Harris, 2002). As some samples with concentration at the detection limit can show fluorescence below the blank reading, there is limited accuracy between the detection limit and the limit of quantitation.

Unhybridized probe had a fluorescence detection limit of 461 and limit of quantitation of 761, which represents a probe detection limit of 3.16×10^{-3} pmoles and a probe limit of quantitation of 1.58×10^{-2} pmoles (Figure 3.1). Samples with *C. jejuni* suspended in PBS showed a detection limit of 3357 and a limit of quantitation of 6509. The detection limit of the FISH procedure can also be reported in terms of CFU/ml, using Figure 3.2. The cell concentration required for a positive signal was found by determining the point at which the samples were read above the detection limit line. This was calculated to be a detection limit of 7.2 $\log_{10}$CFU/ml and a limit of quantitation of 7.5 $\log_{10}$CFU/ml.

3.3.3 Evaluation of enrichment medium

Since the detection limit of the FISH assay was quite high, enrichment would be required to apply FISH to naturally contaminated poultry samples. Growth of a *C. jejuni* isolate was evaluated for four media-types (TSB-Sup, BB-Sup, BB-blood and a semi-solid medium). All media, except the semi-solid aerobic medium (aerobic only), were incubated microaerophilically and aerobically for 48h.

Comparison of the atmospheric conditions showed that *C. jejuni* in both TSB-Sup and BB-Sup reached significantly lower cell concentrations when grown aerobically than grown microaerophilically (Figure 3.3). After 24h of enrichment, the samples grown aerobically were at least 3 $\log_{10}$ lower in cell counts than microaerophilic samples, and after 48h of incubation, the average difference was 2-3.5 $\log_{10}$. It was concluded that the addition of the Campylobacter Growth Supplement to TSB and Bolton Broth only marginally supported aerobic growth of non-stressed *C. jejuni*.

Microaerophilically, BB-Sup had significantly higher cell counts compared to TSB-Sup. After 24h of enrichment, there was a one $\log_{10}$ difference in cell counts and after 48h of enrichment, this difference reached 1.7 $\log_{10}$ (Figure 3.3). Overall, BB-Sup was the most effective enrichment when grown microaerophilically.

The semi-solid aerobic medium was the most effective of the aerobically grown media after 48 hours of enrichment, although there appeared to be no detectable cell counts after 24 hours of enrichment. Cell counts were less than one log below microaerophilically enriched BB-Sup. This ability to enrich *C. jejuni* aerobically would be of great benefit, as it would eliminate the cost and equipment required for the microaerophilic atmosphere. Unfortunately, the agar present in the medium interfered with the centrifugation required to harvest cells after enrichment (data not shown). Thus, the semi-solid medium was not investigated further.

The alternative medium, BB-blood, was also tested both aerobically and microaerophilically. As shown in Figure 3.4, there was no apparent difference between aerobic and microaerophilic cell counts after 24 and 48h of enrichment. Although counts in the medium with blood were 1.8 $\log_{10}$ lower than in BB-Sup, the ability to enrichment aerobically was considered to be more important and BB-blood was therefore tested after spiking with chicken.

This evaluation of the effect of the natural flora present on chicken meat on the growth of *C. jejuni* in BB-blood showed no significant difference between aerobic and microaerophilic cell counts after 24 and 48h, but average counts showed higher variability than in pure culture samples (Figure 3.5). After 24h, aerobically grown samples had higher average cell counts (1.9 $\log_{10}$ difference), but after 48h, microaerophilically grown samples achieved cell counts similar to aerobic samples (0.4 $\log_{10}$ difference). It was concluded that BB-blood was capable of supporting aerobic growth of *C. jejuni* both in pure culture and in the presence of background

flora from chicken meat. A non-inoculated sample of BB-blood spiked with chicken meat was enriched as a control to ensure that the *Campylobacter* detected originated from the inoculum and not from the meat. Plating showed that non-*Campylobacter* flora was present, but no suspect *Campylobacter* colonies were detected.

3.3.4 Fluorescence of sample blanks

Background fluorescence of the FISH procedure was evaluated for different sample blanks: water treated with DEPC, *P. putida* LV 2-4, BB-blood, lysed BB-blood and BB-blood spiked with chicken meat. Comparison of blank sample types showed that DEPC water, *P. putida* LV 2-4 and lysed BB-blood had the lowest fluorescence readings (690 to 1300). They were not significantly different from each other, although DEPC water showed slightly lower fluorescence than the other two sample types (Figure 3.6). Presence of whole blood significantly increased fluorescence levels (28900 to 37500) and chicken meat resulted in slightly higher sample blanks than enrichment broth alone. The removal of blood by lysing with ultrapure water reduced background fluorescence levels similar to DEPC water and *P. putida* LV 2-4.

3.3.5 Removal of blood and background matter prior to FISH

The experiments with naturally contaminated samples combining enrichment in BB-blood and FISH showed that incubation greatly alters the medium such that both sample debris and background fluorescence increase significantly (Figures 3.6 and 3.7). Therefore, different approaches were tested to reduce background fluorescence: lysing of blood after enrichment with and without filtration through a 0.8μm membrane filter, lysing of blood after enrichment and treatment with BDC, and a combination of lysing, BDC and filtration. For all treatments, except

the combination of lysing, BDC and filtration, only a small 1ml volume of spiked sample could be processed once the medium had been incubated for 48h at 42°C. Larger sample volumes became unmanageable due to debris present. For 10ml sample volumes, treatment with lysing, BDC and filtration was the only method that would remove enough debris in order to continue through the FISH procedure (Figure 3.7D). Samples treated with lysing and BDC were manageable, but the level of blank fluorescence was saturated (Figure 3.7B). As shown in Figure 3.8, there was only a small difference in cell counts before and after lysing, with higher counts in lysed samples.

Although, lysing, BDC and filtration through a 0.8μm membrane removed background matter, filtering was found to be difficult and clogging caused problems in some samples. Thus, prefilters were tested as a means of removing larger particles prior to filtering through the 0.8μm membrane filter. Overall, the addition of a prefilter did not affect cell recovery (Figure 3.8), but it did improve the ease of filtering such that clogging was no longer a problem. The prefilter APFB 2500 was chosen as the most effective filter, since the average cell counts after filtering were slightly higher than with the other 3 prefilters.

3.4 Discussion

The *Campylobacter* probe was initially tested to determine its specificity, ensuring that only *Campylobacter*, in particular *C. jejuni,* would be detected. Results of specificity testing showed that the probe hybridized only to *C. jejuni* and none of the non-*Campylobacter* isolates. Although only a limited number of *C. jejuni* could be included, the 21 isolates tested were considered representative of the probe ability. Testing with the non-*Campylobacter* isolates, representing a wide range of bacteria, showed that the probe was able to only hybridize to *C.*

jejuni, as no cross-reactivity was seen. The probe sequence is complementary to 16s rRNA of all *Campylobacter* spp. and it is thus probable that hybridization would also occur with species other than *C. jejuni*. This is advantageous, as any species of *Campylobacter* can cause campylobacteriosis, but *C. jejuni* was targeted as it has been linked to a number of sequelae in campylobacteriosis patients.

To date, all published FISH protocols require a step to adhere the sample to glass slides prior to performing FISH, followed by microscopy. This current study developed a protocol that can be performed with 1ml volumes in solution which not only reduces the time required for completing the test, but also the amount of reagents and buffers needed for each sample. In addition, the FISH signal can be analysed by microscopy as well as automated systems such as a fluorescence plate reader or flow cytometry.

As shown in Figure 3.2, the detection limit of unhybridized probe is 3.16×10^8 pmol, which represents 1.90×10^9 molecules of probe that would be capable of hybridizing to rRNA strands. The theoretical detection limit of hybridized *C. jejuni* is dependent on the number of rRNA molecules in each cell. According to Coşkuner et al (2002), a healthy bacterial cell contains an average of 10^3 to 10^5 rRNA strands. As 1.90×10^9 rRNA molecules represent a positive signal and an average of 10^4 rRNA molecules are present in each cell, the theoretical detection limit for *C. jejuni* would be 1.90×10^5 CFU. This theoretical detection limit is based on the assumption that each probe molecule hybridizes with each rRNA molecule present in the cells. This is obviously not the case as the actual detection limit for *C. jejuni* was 100-fold higher with 3.0×10^7 CFU. This difference may be due to lower numbers of rRNA strands present in each cell or, as the procedure is performed *in situ,* it is likely that the probe molecules

will not hybridize to all rRNA strands in the cells, therefore requiring a higher cell concentration for a positive signal.

Experiments with pure *C. jejuni* cultures suggested that cell counts above 7.5 – 8.0 $\log_{10}$CFU/ml are required for a positive detection. With such a high detection limit, an enrichment step was required to use the FISH protocol with naturally contaminated poultry samples, which are commonly contaminated with low levels of *C. jejuni*. For example, Josefsen et al (2004) reported that *C. jejuni* is present at levels of 2.5-4.4 $\log_{10}$CFU/ml. Josefsen et al (2004) suggested that with the development of new hygienic techniques and procedures the need for an enrichment step is becoming a necessity for most detection methods used in food microbiology.

To reduce the cost of enrichment, several enrichment media were examined, with the objective to identify an aerobic enrichment protocol not requiring blood. A blood-free aerobic medium would reduce the cost of enrichment by eliminating the need for blood and specialized equipment used to produce the microaerophilic environment. Jeffery et al (2000) designed a blood-free semi-solid medium that would support the growth of *C. jejuni* when incubated aerobically. The authors showed that the medium was capable of recovering low levels of inoculum, as they are commonly found in naturally contaminated samples; however, they did not determine cell concentrations reached after enrichment and only reported whether the organisms were present on plates inoculated after enrichment. This current study demonstrated that the medium allows for the growth of non-stressed cells from 100 CFU to levels as high as 8.10 ± 0.12 $\log_{10}$CFU/ml (Figure 3.3). Unfortunately, the aerobic semi-solid medium, could not be used for enrichment prior to FISH, as the agar present interfered with centrifugation and other preparation steps, such as BDC.

Alternative media tested included Bolton Broth and TSB with the addition of Campylobacter Growth supplement and Bolton Broth with 5% blood. The Campylobacter Growth supplement was added to ensure that TSB and Bolton Broth contained the necessary nutrients for the fastidious nature of *Campylobacter*. Microaerophilically, both media supported growth of *C. jejuni* 16-2R, but Bolton Broth as the base medium was more effective (Figure 3.3). Josefsen et al (2004) found similar growth of *Campylobacter* in blood-free Bolton Broth when the medium was used for the enrichment of poultry samples prior to detection with PCR. The authors tested three types of blood-free enrichment media, including Preston Broth, Mueller-Hinton Broth and Bolton Broth. Although all three media were able to enrich 5 strains of *C. jejuni* to cell concentrations greater than 8 $\log_{10}$CFU/ml, Bolton Broth was found best to support the growth of low initial cell numbers. Overall, Josefsen et al (2004) suggested that blood-free Bolton Broth was the most effective of the three enrichment media due to the ability to enrich several *Campylobacter* spp. initially present at relatively low cell counts. These results are also supported by Sails et al (2003) who used blood-free Bolton Broth to enrich naturally contaminated poultry samples prior to PCR detection. Of the 26 positive samples, only 7 samples had cell counts between 4-7 $\log_{10}$CFU/ml, with the remaining samples reaching cell counts greater than 7 $\log_{10}$CFU/ml. As with Josefsen et al (2004), blood-free Bolton Broth was shown to support the growth of several *Campylobacter* spp. to high cell concentrations.

Bolton Broth with 5% blood is the current standard medium used by the USDA for the enrichment of *Campylobacter*. Testing laboratories enrich samples microaerophilically, but it has been proposed that Bolton Broth with 5% blood is capable of recovering *Campylobacter* aerobically (Oxoid Ltd.). In the present study, comparison of cell concentrations after enrichment showed no significant difference ($p=0.998$) between microaerophilic and aerobic

incubation, as counts for both samples reached 7-8 $\log_{10}$CFU/ml. These results contrast those of Blais et al (1999) who examined the enrichment abilities of five types of media, two of which were incubated aerobically. For two of three *C. jejuni* serotypes tested, both aerobic media, including Bolton Broth with 5% blood, did not show cell counts comparable to those achieved with microaerophilic incubation. The authors concluded that aerobic growth of *C. jejuni* in a medium may be serotype dependent and suggested that aerobic enrichment of naturally contaminated samples may not be advisable as they can harbour different serotypes. Nevertheless, the present study examined the ability of aerobic enrichment of *C. jejuni* inoculated in BB-blood spiked with chicken meat. The presence of background organisms present on chicken meat may affect the growth of *C. jejuni* due to competition effects. As shown in Figure 3.5, the atmosphere of incubation did not significantly affect cell counts at both 24 and 48h. Aerobically incubated samples had slightly higher cell counts after 24h, which may allow the *Campylobacter* to become established before the growth of background flora. Growth of *C. jejuni* in BB-blood, microaerophilically and aerobically showed no significant difference between non-spiked samples and those spiked with chicken meat ($p=0.141$ and $p=0.187$, respectively). Although microaerophilic BB-Sup showed higher cell counts after 48h, the ability of BB-blood to enrich samples aerobically could be beneficial for routine testing. Thus, both media types could be used with FISH testing.

Application of FISH to *C. jejuni* in different enrichment media showed that blood present in a medium significantly increased background fluorescence, although background bacteria from chicken meat did not appear to greatly affect overall background fluorescence (Figure 3.6). Such apparent autofluorescence can occur in samples containing inorganic or biological matter, particularly blood cells and tissue samples with elastin and collagen (Moter and Göbel, 2000).

Alternatively, non-specific binding of probe may be the reason for the high background fluorescence. Samples grown in a blood medium would therefore require treatment steps to reduce background fluorescence prior to FISH testing. Several methods for removing background fluorescence have been reported, including paraformaldehyde, irradiation and computer programs which subtract autofluorescence from the desired fluorescence. There are several disadvantages to these methods, as chemical treatments can affect fluorescence intensity of the probe and computer programs may generate false positive results (Neumann and Gabel, 2000). Treatment steps used in the current study included filtering, lysing of blood with ultrapure water and BDC, all of which do not involve the use of strong chemicals that could affect the probe fluorescence.

The addition of a lysing step allowed the blood to be removed, therefore reducing the fluorescence observed with whole blood cells (Figure 3.6). Lysis of blood cells also resulted in a slight increase in cell concentration. This may be due to the fact that *C. jejuni* cells adhering to red blood cells during enrichment are released during the lysing step. Thus, there would be more free cells present capable of forming colonies when plated, which could explain the slight increase in cell counts.

In conclusion, the *Campylobacter* probe tested proved to be specific for the detection of *Campylobacter* spp. by FISH. As a high cell count was required for the detection of a positive signal using a fluorescence plate reader, analysis of a large sample volume after enrichment would be beneficial. Evaluation of different enrichment media showed that Bolton Broth with *Campylobacter* Growth Supplement, incubated microaerophilically or Bolton Broth with blood incubated either microaerophilically or aerobically, were suitable choices. However, the blood required for optimal growth during enrichment hampered the detection of the FISH signal due to

non-specific fluorescence or debris interfering with the FISH protocol. Different approaches to eliminate this problem were evaluated. Lysing the blood followed by BDC and filtration through a prefilter and 0.8μm membrane filter resulted in a sample that could be processed through FISH, showing reasonably low background fluorescence.

Table 3.1 Results of probe specificity testing using 22 *C. jejuni* isolates.

C. jejuni strain	Total cell staining with DAPI	FISH signal with *C. jejuni* probe
16-2R	+	+
B99-1158	+	+
B99-796	+	+
B99-189	+	+
551	+	+
554	+	+
557	+	+
610	+	+
614	+	+
620	+	+
627	+	+
636	+	+
647	+	+
649	+	+
643	+	+
706	+	+
708	+	+
712	+	+
722	+	+
723	+	+
724	+	+

+ = fluorescence signal detected

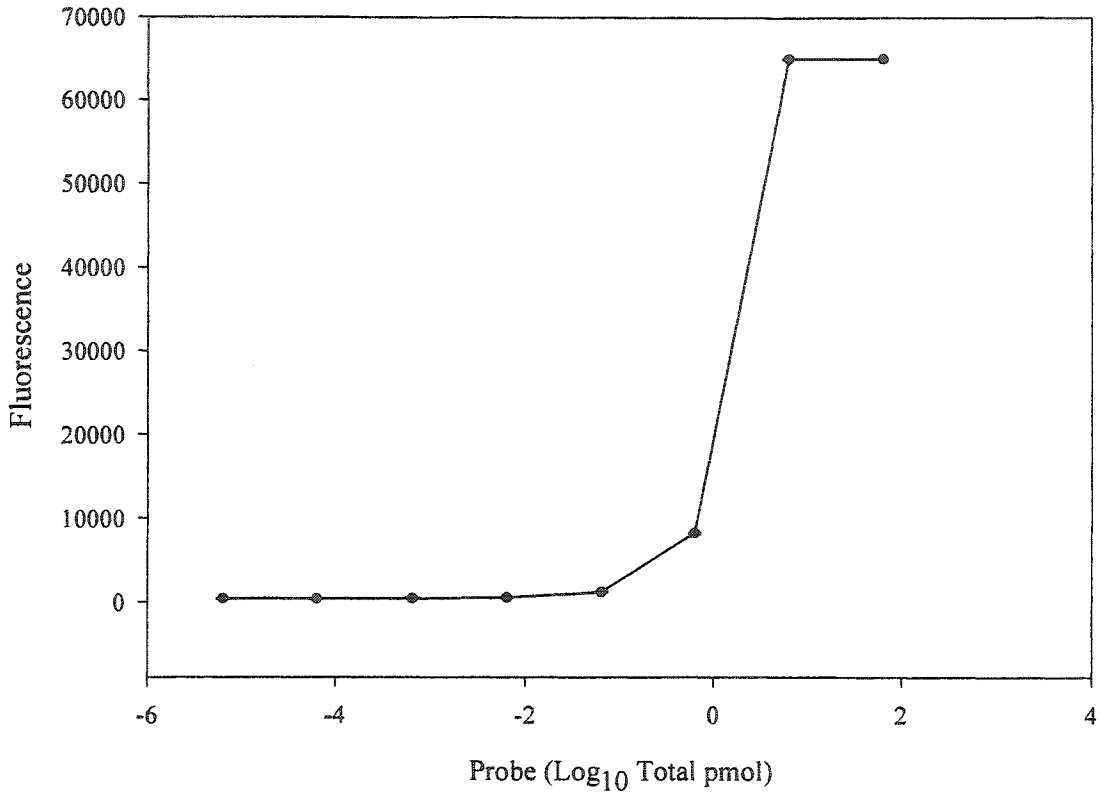
Table 3.2: Results of cross-reactivity testing using 16 non-*Campylobacter* isolates.

Non-*Campylobacter* Isolate	Total cell staining with DAPI	FISH signal with *C. jejuni* probe
Alcaligenes faecalis ATCC 35655	+	-
Citrobacter freundii ATTC 8090	+	-
Escherichia coli ATTC 25922	+	-
Klebsiella pneumonia ATTC 13883	+	-
Proteus mirabilis ATTC 43071	+	-
Proteus vulgaris ATCC 13315	+	-
Pseudomonas fluorescens ATTC 49838	+	-
Pseudomonas putida LV2-4	+	-
Serratia marcescens ATTC 14756	+	-
Bacillus subtilis ATTC 6633	+	-
Clostridium perfringes ATTC 13124	+	-
Micrococcus luteus ATCC 9341	+	-
Staphylococcus epidermidis ATTC 12228	+	-
Saccharomyces cerevisiae ATTC 2601	+	-

+ = fluorescence signal detected
- = no fluorescence signal detected

Figure 3.1 Detection limit and limit of quantitation for unhybridized probe.

A.



B.

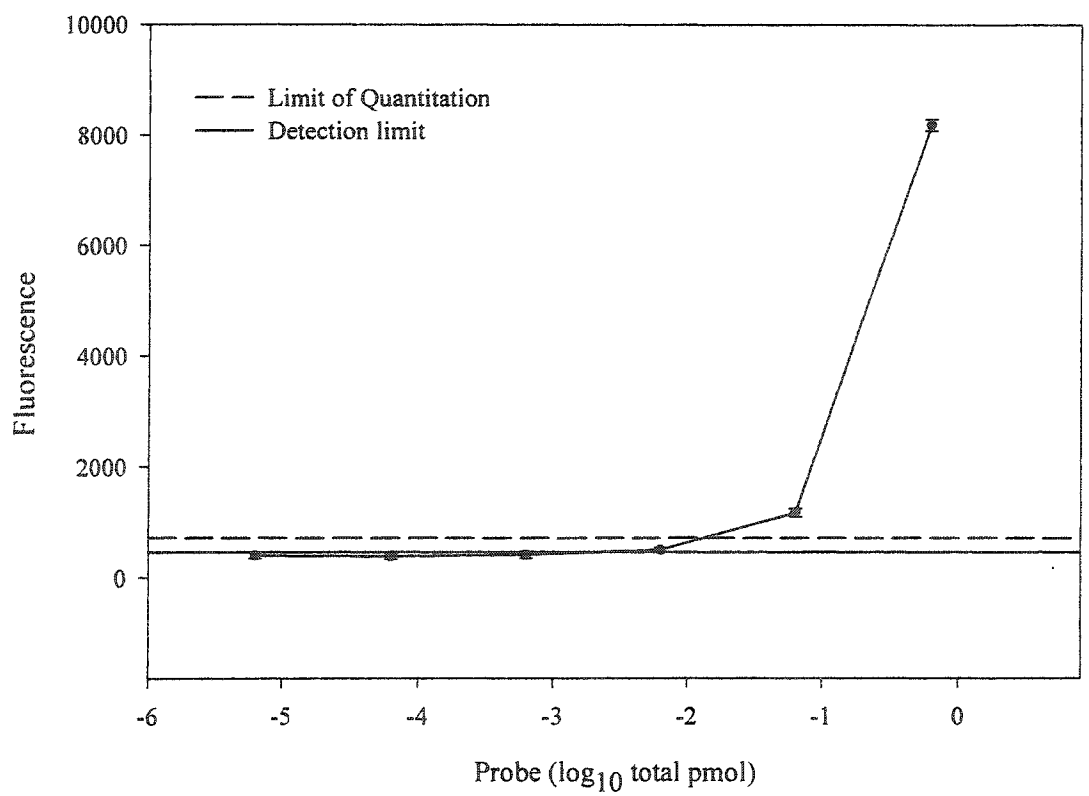


Figure 3.2 FISH signal of *C. jejuni* suspended in PBS.

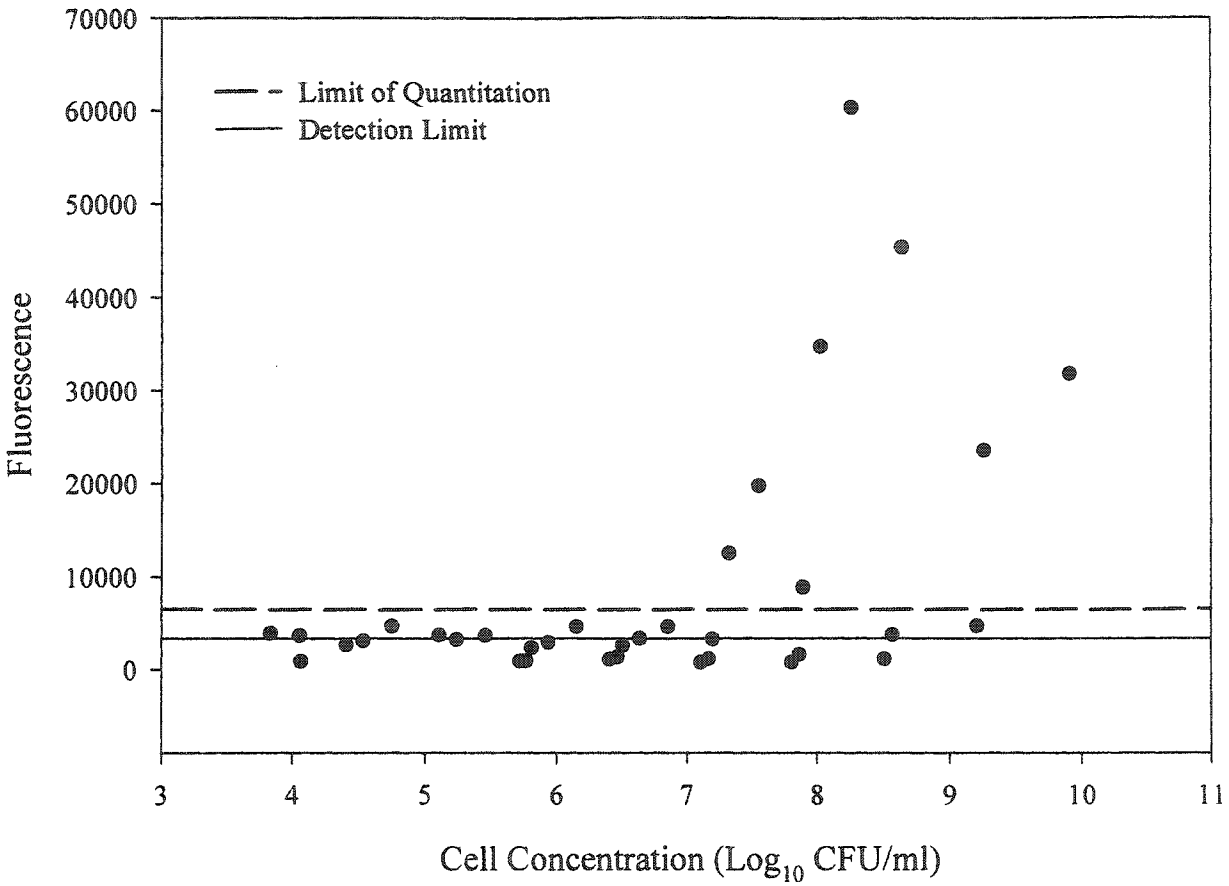
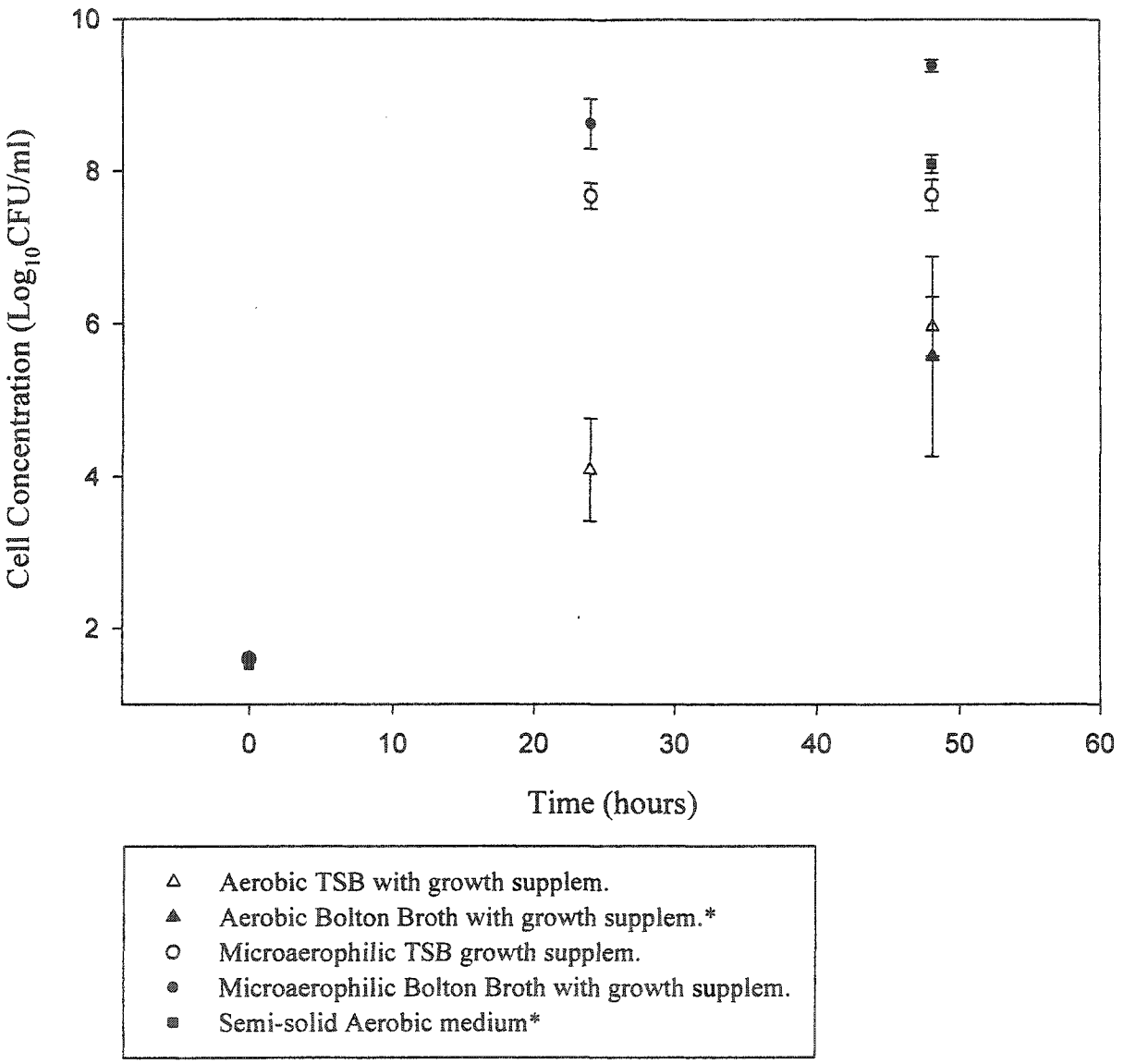


Figure 3.3 Comparison of aerobic and microaerophilic growth of *C. jejuni* 16-2R in different enrichment media.



* No detectable growth at 24h time point (<20 CFU/ml)

Figure 3.4 Growth of *C. jejuni* in Bolton Broth with 5% blood.

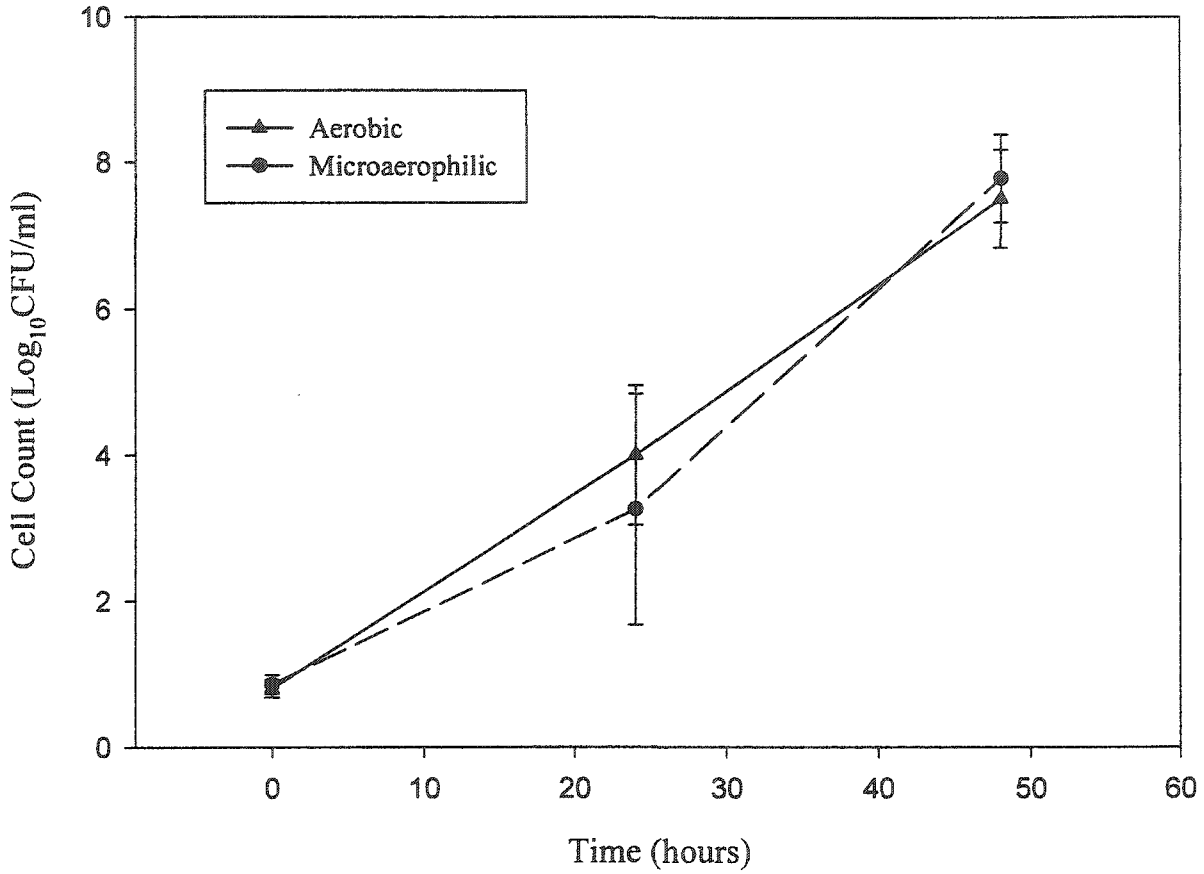


Figure 3.5 Growth of *C. jejuni* in Bolton Broth with 5% blood spiked with chicken meat.

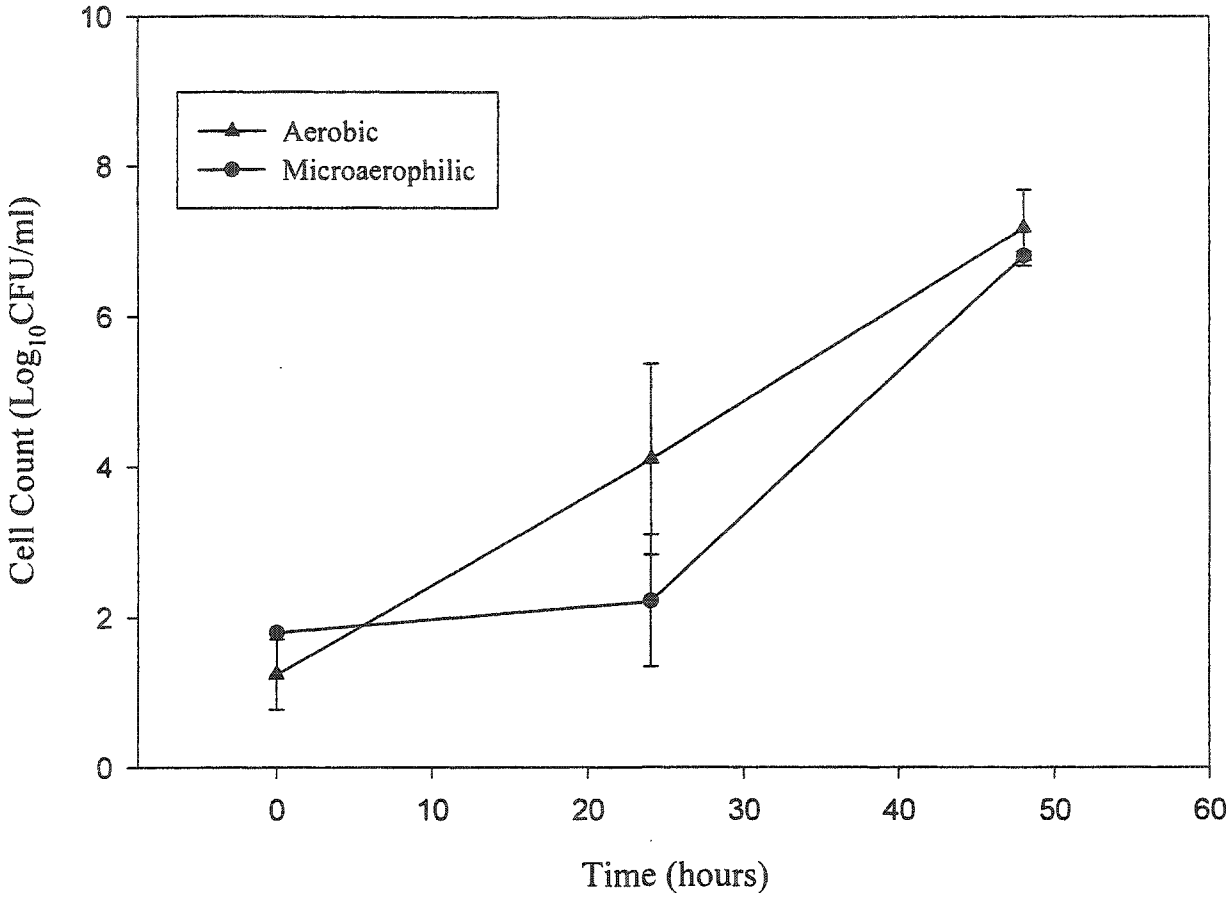


Figure 3.6 Average fluorescence of sample blanks detected by the fluorescence plate reader.

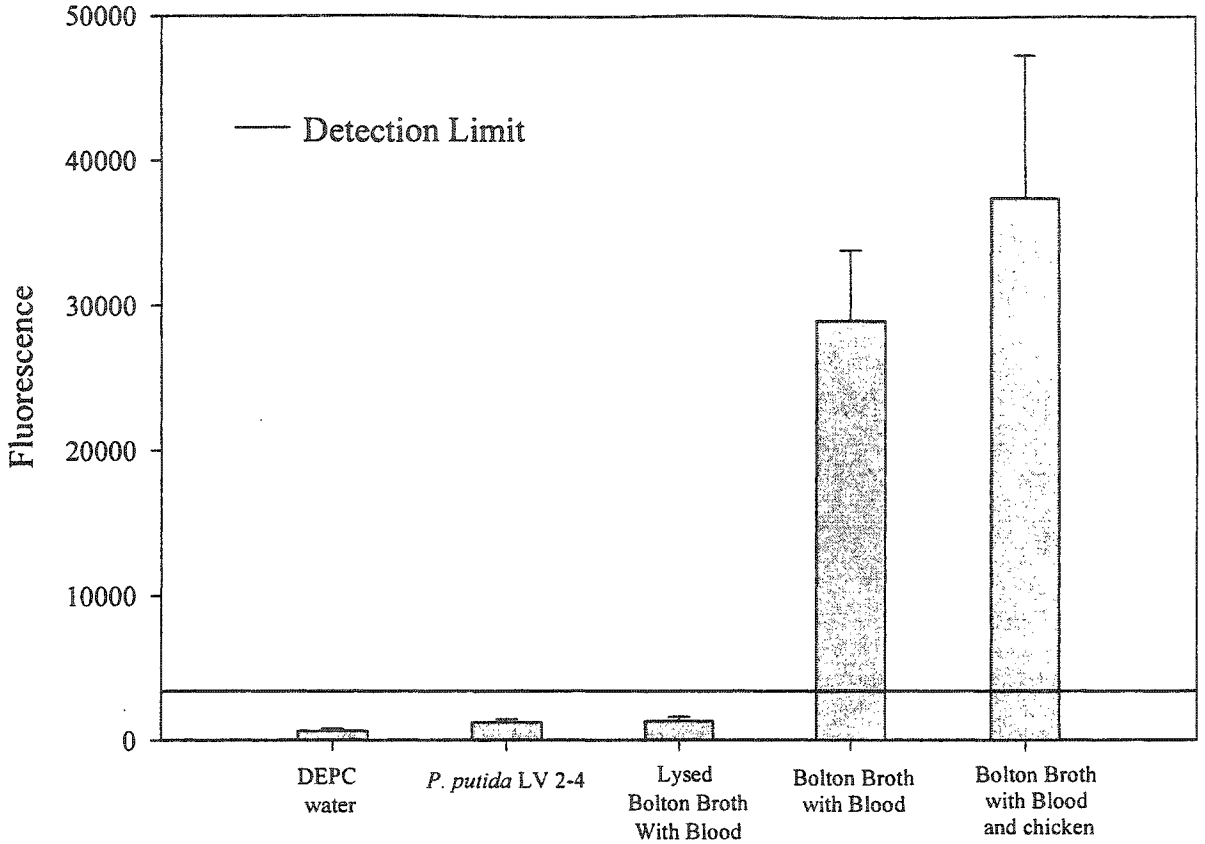


Figure 3.7 Fluorescence of 1ml and 10ml volumes of blank and sample readings with preparation steps prior to FISH testing.

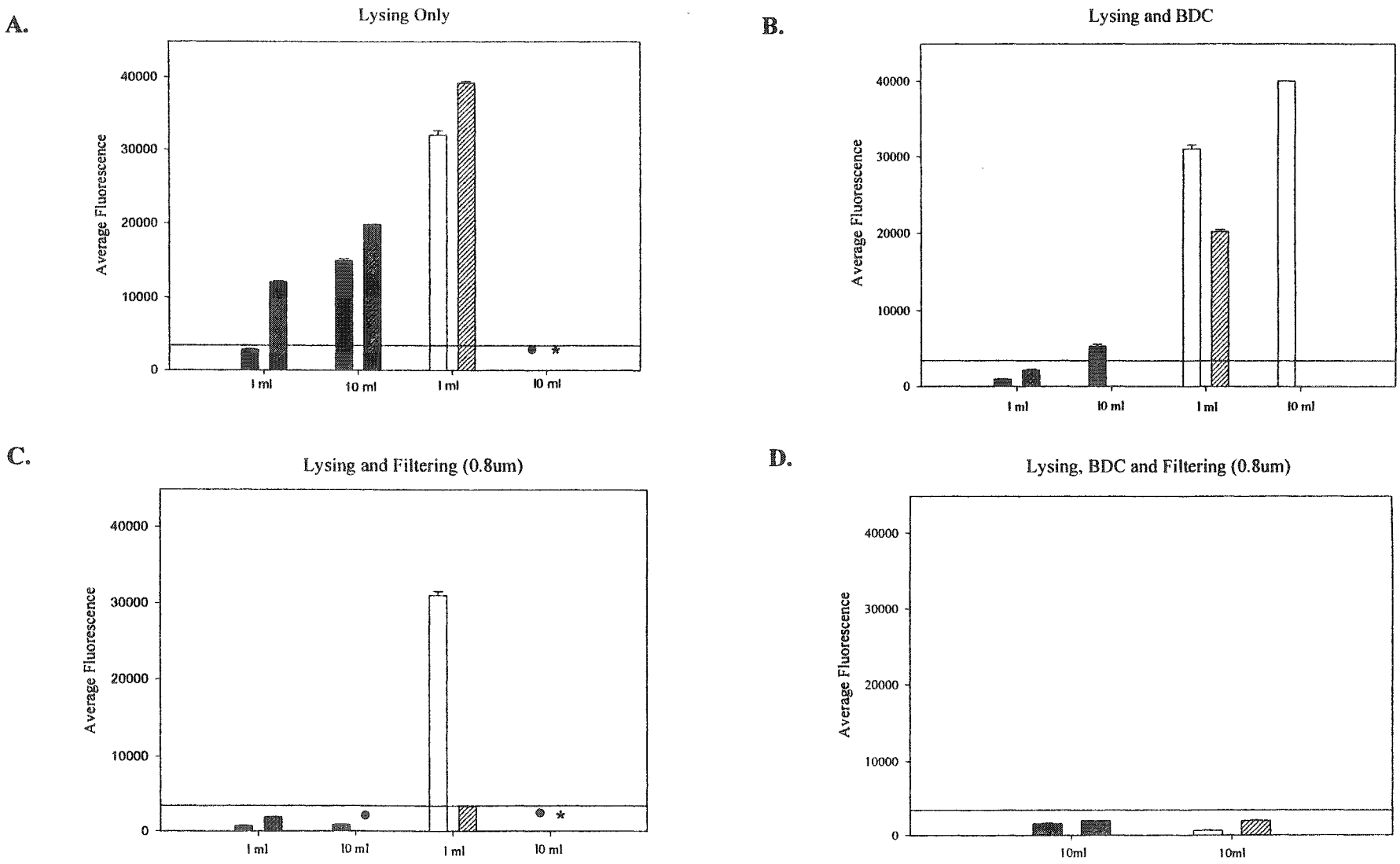
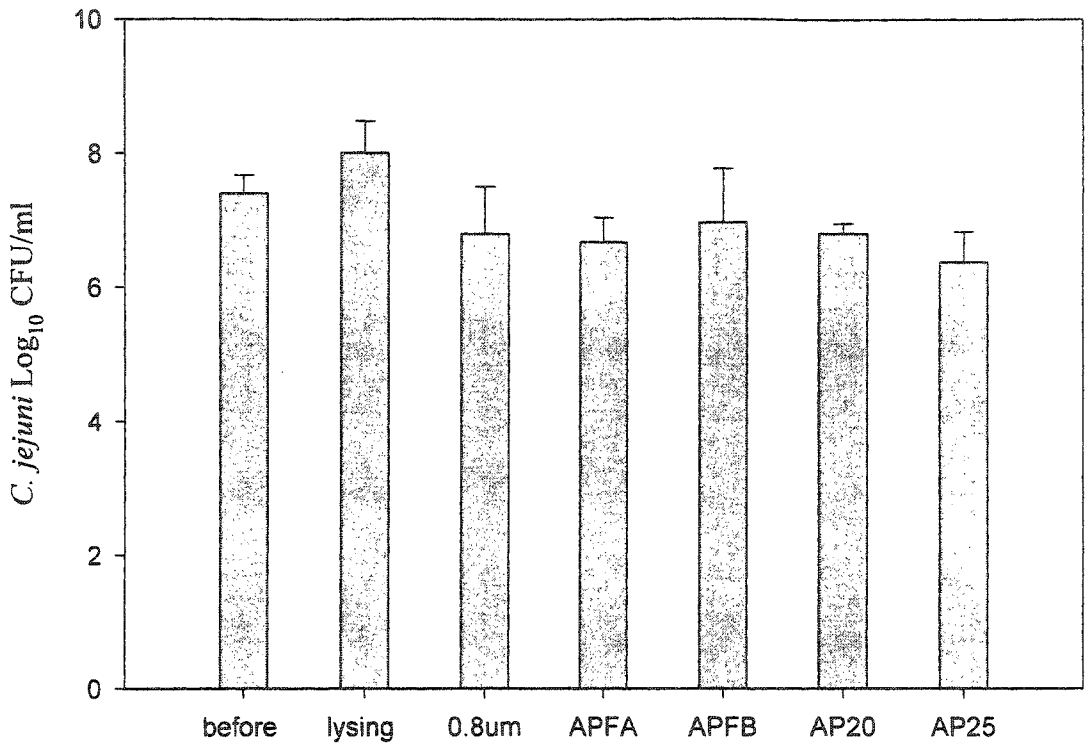


Figure 3.8 Evaluation of cell recovery due treatment steps prior to FISH testing.



Legend

Before = Cell Concentration Prior to treatment steps
Lysing = Cell Concentration after lysing of blood cells with ultrapure water
0.8µm = Cell Concentration after BDC and filtration through a 0.8µm filter
APFA = Cell Concentration after BDC and filtration with a APFA02500 prefilter
APFB = Cell Concentration after BDC and filtration with a APFB02500 prefilter
AP20 = Cell Concentration after BDC and filtration with a AP2002500 prefilter
AP25 = Cell Concentration after BDC and filtration with a AP2502500 prefilter

4. Detection of *Campylobacter* in naturally contaminated poultry.

4.1 Introduction

Campylobacter spp., as outlined in Chapter 1, is most commonly associated with raw or undercooked poultry, leading to changes in approaches to food safety throughout the poultry industry. The industry has begun to implement new methods aimed at eliminating or at least reducing the prevalence of the organisms. Currently, poultry products worldwide can not be guaranteed to be free of *Campylobacter* spp., with many countries having a prevalence as high as 80% (Chapter 1, Table 1.1).

All poultry processing facilities routinely monitor their process for the presence of selected microorganisms in order to identify problematic areas and to ensure that processes designed to control contamination are working effectively. This can help to reduce the prevalence and level of contamination with pathogenic bacteria. The current standard for the detection of *Campylobacter* spp. is the conventional method of enrichment in a selective medium, followed by plating on a selective medium and biochemical confirmation of suspect isolates (Chapter 1, Section 1.5.1). There are several disadvantages to this approach, but until a more effective method is proven, it will remain the standard. As outlined in Chapter 1 (Figure 1.2) a typical test for *Campylobacter* spp. can take up to nine days before a positive result can be determined. After such a long time many products will have been processed and distributed to either the consumers or to another area for further processing. This problem could be greatly reduced using the FISH procedure. Even with the addition of a 48h enrichment step, the presence of *Campylobacter* spp. could be determined within only 3 days, compared to the current nine days. This would not only decrease the period between sampling and final confirmation, it would also reduce cost by eliminating the need for biochemical tests and selective plate media.

The objectives of this chapter are to evaluate the FISH protocol for the detection of *C. jejuni* in naturally contaminated poultry samples, which include both whole-carcass rinse samples and live-bird swabs. Since enrichment is required prior to FISH, three types of enrichment media are also included, namely, microaerophilic enrichment in Bolton Broth with blood and in Bolton Broth with *Campylobacter* Growth supplement and aerobic enrichment in Bolton Broth with blood. For the latter two media, results obtained by conventional plating will be compared to positives determined with FISH. The first enrichment protocol serves as the "gold standard" since it is the method currently used by the USDA.

4.2 Materials and Methods

4.2.1 Collection and transportation of samples

Naturally contaminated poultry samples were collected in a commercial poultry slaughtering plant in Southern Ontario. A total of 18 whole carcass rinse samples and 18 live bird swabs were tested. Whole carcass rinses were prepared by placing an individual carcass into a large stomacher bag with 500ml of sterile peptone water. After vigorous shaking, the liquid was removed and shipped to the laboratory. Live bird swabs were taken by rubbing a wetted sample sponge (approx. 5cm x 10cm) over an individual bird. The sponge was then placed in a stomacher bag and shipped to the laboratory. Samples were shipped on ice and received in the laboratory within 24h of collection.

4.2.2 Sample preparation

The peptone water (approx. 400ml) from each whole carcass rinse was divided into two 250ml sterile centrifuge bottles and spun for 15 minutes at 10,500 x g. The supernatant was poured off and the pellets were resuspended and combined into 10ml sterile peptone water.

The sample sponges used for the live bird swabs were aseptically cut into pieces and placed with 125ml sterile peptone water into a stomacher bag. The bag was then pulsifed for 2 minutes. The peptone water was pipetted into a 250ml sterile centrifuge bottle and spun for 15 minutes at 10,500 x g. The supernatant was removed and the pellet resuspended in 10ml sterile peptone water.

4.2.3 Enrichment

Each sample was enriched in three different conditions: i) Bolton Broth with blood (BB-blood), microaerophilic, ii) BB-blood, aerobic and iii) Bolton Broth with growth supplement (BB-Sup), microaerophilic. Each medium was prepared according to Chapter 3 (Section 3.2.6.1) with the addition of Bolton Broth Selective Supplement (SR 183, Oxoid). For each 10ml sample, prepared as described above (Section 4.2.2), 3ml aliquots were pipetted into each enrichment broth.

4.2.4 Detection of Campylobacter using FISH

For all samples enriched aerobically in BB-blood, a 10ml volume was treated with lysing, BDC and filtering through an APFB2500 prefilter and 0.8μm filter as outlined in Section 3.2.8.6. Due to the presence of blood cells in the whole-carcass rinse samples enriched in BB-Sup, 10ml sample volumes were treated with lysing and BDC as outlined in Section 3.2.8.4. For live-bird

swabs enriched in BB-Sup, 10ml of sample was centrifuged for 10 minutes at 3,200 x g. The supernatant was poured off and the pellet resuspended in 1ml of 4% paraformaldehyde. All samples were then treated with the FISH procedure and examined in the fluorescence plate reader (Sections 3.2.3). Samples were considered positive if the detection limit of 1962 was exceeded for samples enriched aerobically in BB-blood and a detection limit of 3357 was exceeded for samples enriched in BB-Sup.

4.2.5 Detection of Campylobacter with conventional plating method

In addition to FISH, all enrichment media were spiral plated to identify the presence of suspect *Campylobacter* spp. and to determine cell counts of suspect *Campylobacter* colonies, and non-*Campylobacter* background flora. Samples were plated on modified *Campylobacter* Agar with mCCDA selective supplement (SR10183, Oxoid) and incubated microaerophilically, at 42°C for 48h, (Hunt et al, 1998).

For plates that showed growth of typical *Campylobacter*, two suspect colonies were streaked onto mCCDA plates with mCCDA selective supplement and incubated at 42°C for 48h, microaerophilically. Pure cultures were then gram-stained, evaluated for motility and subjected to biochemical tests (Table 1.2, Hunt et al, 1998). Gram-negative, small and motile rods that showed positive results for the nitrate reduction test, catalase and oxidase reactions and a negative result for glucose fermentation were considered *Campylobacter* spp. The hippurate test was used to distinguish *C. jejuni* from all other *Campylobacter* spp. For some isolates, the positive biochemical test results were confirmed with the Dryspot *Campylobacter* test (Oxoid), which shows a positive reaction for latex agglutination of enteropathogenic *Campylobacter*.

4.3 Results

4.3.1 Comparison of Enrichment media

Overall, *Campylobacter* spp. was detected in 64% of the 36 samples tested (Table 4.1). Live-bird swab samples showed a higher prevalence (78%) than whole-carcass rinse samples (50%). Results of biochemical testing showed that 13 of the 23 samples were contaminated with *C. jejuni,* a prevalence of 56%, with the remaining positive samples containing *Campylobacter* spp. (Tables 4.2 and 4.3). Microaerophilically grown BB-blood and BB-Sup performed similarly well. In comparison, aerobic BB-blood was less consistent, with the least number of positive samples (Table 4.1). It should be noted however, that in four of the samples (3 live-bird swabs (15S, 16S and 18S) and one whole-carcass rinse (22W)), the positive result was only obtained in aerobic BB-blood (Tables 4.2 and 4.3).

The number of positives in carcass rinse samples was much lower than for live-bird swabs (Table 4.1). Positive results for carcass rinse samples were not consistently found in each enrichment medium. Only two samples (15W and 16W) were positive for all three types of enrichment, one sample (11W) was positive for 2 media, with the remaining samples being positive for only one medium (Table 4.2). For live bird swabs, 5 positive results were found in all three media types, 3 were positive in 2 media and 6 positive results were found in only one medium (Table 4.3).

Of the 36 samples tested, aerobic BB-blood had 4 samples in which no growth was detected compared to the 9 samples that were enriched microaerophilically in BB-Sup. Overall, aerobic BB-blood showed considerably lower cell counts for both *Campylobacter* and background flora (Table 4.2 and 4.3). Average *Campylobacter* spp. counts were $\log_{10}$ 7.1± 0.9 and average background flora was $\log_{10}$ 6.1± 1.6. Microaerophilic BB-blood and BB-Sup

showed higher average counts for both *Campylobacter* (log_{10} 8.4 ± 1.1 and log_{10} 8.5 ± 1.1, respectively) and background flora (log_{10} 7.3 ± 1.9 and log_{10} 8.2 ± 1.7, respectively).

4.3.2 Evaluation of detection with FISH

For samples enriched aerobically in BB-blood, the results of FISH testing showed significantly less positive samples (Table 4.4a). Of the two samples found positive with FISH, only one (15W) was also positive with the conventional plating method. The one false positive sample found with FISH (12W), had also the highest counts for background flora (Table 4.2).

In contrast, samples incubated in BB-Sup showed 29 positive samples with FISH, but only 15 positive samples with the conventional plating method (Table 4.4b). Three of these samples (18W, 21W and 18S) did not have detectable growth, but were positive with the FISH procedure, while the remaining 11 samples showed background flora (Tables 4.2 and 4.3). Thus, the presence of background flora seemed to increase the fluorescence of the sample, resulting in false positives. However, 7 of these false positives did show growth of *Campylobacter* spp. in one of the other enrichment media.

4.4 Discussion

The study not only evaluated the detection of *Campylobacter* spp. using FISH, but also three types of enrichment media. Results of the conventional plating method showed 64% of samples were contaminated with *Campylobacter* spp. As outlined in Chapter 1 (Table 1.1), this is not uncommon, as prevalence in poultry can be as low as 45% and as high as 88%. Of the samples positive for *Campylobacter* spp., 56% were determined to be *C. jejuni*, which is below the 80-100% observed in several other countries, although no prevalence comparison could be

made to a North American country (Chapter 1, Table 1.1). The remaining positive samples could be only classified as *Campylobacter* spp., but studies have shown that *C. coli* would be the next leading species isolated from poultry (Park, 2002).

A comparison of the three enrichment media showed that, microaerophilically incubated media were the most effective. Although, aerobic Bolton Broth with blood showed potential for enrichment in preliminary studies, it was not as efficient with naturally contaminated samples, as fewer positive samples were detected and *Campylobacter* concentrations reached after enrichment were significantly lower. It may be possible that samples found negative did contain *Campylobacter* spp., but were out-competed or overgrown by background flora. The lower *Campylobacter* concentrations and less positive samples are consistent with results found by Blais et al (1999) who demonstrated that aerobic incubation showed significantly lower cell counts and the ability to culture *Campylobacter* spp. aerobically was serotype dependent. This reduces the usefulness since poultry samples can be contaminated with more than one species or strain. Overall, aerobic Bolton Broth with blood can not be considered an ideal medium for the enrichment of *Campylobacter* spp. in naturally contaminated poultry samples.

Bolton Broth with *Campylobacter* Growth supplement and microaerophilic Bolton Broth with blood not only showed more positive samples, but *Campylobacter* reached much higher counts than in aerobically incubated samples. Thus, it is better to enrich for *Campylobacter* spp. in optimal atmospheric conditions, rather than mimicking the microaerophilic environment by using a tightly capped container, as suggested by Oxoid, Ltd.. The results also indicate that the blood supplement could be replaced by a simple growth supplement. The ability to enrich *Campylobacter* spp. samples in blood-free Bolton Broth has been previously demonstrated by Sails et al (2003) and Josefsen et al (2004), who used Bolton Broth without the addition of any

supplement. Both authors found cell counts similar to the current study, showing that the medium could be used in place of the standard medium containing blood. BB-Sup was equal to BB-blood in terms of enrichment capabilities, but the blood-free medium would be more beneficial as the blood inhibits many molecular-based techniques.

The ability to detect *Campylobacter* spp. using the FISH procedure was dependent upon the medium used for enrichment. Only two positive samples were found with FISH when samples were enriched aerobically, significantly lower than the 12 samples positive with conventional plating (Table 4.4a). As shown in Tables 4.2 and 4.3, *Campylobacter* cell counts in the false negative samples were below the detection limit of $\log_{10}7.33$, determined in Chapter 3 (Figure 3.2). This may explain the lack of a positive probe signal. In comparison, the results of FISH testing of samples enriched in BB-Sup showed numerous positive results, many of which correlated to the conventional plating method. This may be due to higher *Campylobacter* counts which were generally above the detection limit of FISH. As several of the samples showed only non-*Campylobacter* on plates grown from BB-Sup, it was thought that there were possibly *Campylobacter* cells present, but they were overgrown by the background flora. Therefore, the FISH results from BB-Sup were compared to positive samples detected in any of the enrichment media. From this it was determined that 20 of the 23 positive samples were detected with both FISH and plating. However, 10 samples were positive with FISH, but no *Campylobacter* were found in any of the three media. It is possible that *Campylobacter* spp. were present in the enrichment medium, but were overgrown by background flora on the plate medium. Alternatively, the non-*Campylobacter* flora present may have been the cause for fluorescence. These two theories can possibly explain the 8 of 10 samples that showed bacterial growth and positive fluorescence, but it does not explain the two samples that showed positive results for

FISH and no detectable growth by plating. The fluorescence of these two samples may be due to sample particles, which were not removed by BDC and may have increased the background fluorescence. Another possibility is that background flora was present in the enrichment medium, but may not have been culturable on the plates because of the additional antibiotics used to increase selectivity. In order to evaluate this theory, one would have to plate the enrichment medium on a non-selective plate.

Overall, the FISH procedure seems capable of detecting *Campylobacter* spp. when cell concentrations are above the detection limit, which in turn is dependent upon the type of enrichment medium used. It was concluded that the most effective media were those incubated microaerophilically, and that blood is not the only supplement that can be used to support the growth of *Campylobacter*. As high *Campylobacter* counts were found when BB-blood was enriched microaerophilically, it is possible that the FISH procedure would also show positives for these samples.

Table 4.1 Detection of *Campylobacter* spp. in different enrichment media.

Sample Type	Aerobic BB-blood[a]	Microaerophilic BB-blood	BB-Sup[b]	Any of the three media
Whole carcass rinse	4 / 18[c]	5 / 18	6 / 18	9 / 18
Live-bird swab	8 / 18	10 / 18	9 / 18	14 / 18
Total	12 / 36	15 / 36	15 / 36	23 / 36

a = Bolton Broth with blood
b = Bolton Broth with *Campylobacter* Growth supplement
c = positive samples / number of samples tested

Table 4.2 FISH results and cell counts of *Campylobacter* spp. and non-*Campylobacter* in whole-carcass rinse samples.

Sample	Bolton Broth with 5% blood, microaerophilic		Bolton Broth with 5% blood, aerobic			Bolton Broth with Campylobacter Growth supplement, microaerophilic		
	Campylobacter spp. (log_{10}CFU/ml)	Background flora (log_{10}CFU/ml)	*Campylobacter* spp. (log_{10}CFU/ml)	Background flora (log_{10}CFU/ml)	Fluorescence[a]	*Campylobacter* spp. (log_{10}CFU/ml)	Background flora (log_{10}CFU/ml)	Fluorescence[b]
7W	8.53	-[c]	-[c]	7.21	- (938)	-[c]	9.17	+ (59758) ‡ †
8W	-[c]	8.62	-	7.55	- (678)	9.20*	-[c]	+ (47307)‡
9W	8.89*	-	-	6.00	- (1207)	-	9.17	+ (27474) ‡ †
10W	-	8.77	-	6.45	- (1010)	9.27	-	+ (65000)‡
11W	8.75	-	-	7.28	- (929)	8.06	-	+ (4460)‡
12W	-	8.76	-	8.56	+ (19027)	-	9.04	+ (22702) †
13W	ND	ND	ND	ND	- (1102)	ND	ND	- (1598)
14W	ND	ND	ND	ND	- (711)	-	8.66	+ (10404) †
15W	9.39	-	7.33	-[c]	+ (3284)	8.33	-	+ (5214)‡
16W	9.46*	-	6.92*	-	- (1070)	9.33*	-	+ (26870)‡
17W	-/-[d]	6.24	ND	ND	- (891)	-/-[d]	5.50	+ (10820) †
18W	-/-	6.76	-/-[d]	7.06	- (1007)	ND	ND	+ (19928) †
19W	-/-	8.20	-/-	2.25	- (1441)	ND	ND	- (2942)
20W	-/-	8.71	-/-	6.41	- (1040)	-/-	8.51	+ (28518) †
21W	-/-	6.20	-/-	6.82	- (959)	ND	ND	+ (16024) †
22W	ND	ND	6.20*	-	- (1371)	ND	ND	- (3238)
23W	-/-	6.30	-/-	4.12	- (1107)	6.83*	-	+ (43889)‡
24W	-/-	5.50	-/-	4.44	- (1593)	-/-	4.38	+ (17492) †

ND = no growth detected

a) + = fluorescence above detection limit of 1962; - = fluorescence below detection limit

b) + = fluorescence above detection limit of 3357; - = fluorescence below detection limit

c = no background flora or no suspect *Campylobacter* spp. colonies

d = confirmed to be non-*Campylobacter* spp. colonies

* = *C. jejuni*

† = positive with FISH, but no *Campylobacter* spp. found on plates from enrichment in BB-Sup.

‡ = positive with FISH, but *Campylobacter* spp. positive on plates from any enrichment

Table 4.3 FISH results and cell counts of *Campylobacter* spp. and non-*Campylobacter* in live bird swab samples.

Sample	Bolton Broth with 5% blood, microaerophilic		Bolton Broth with 5% blood, aerobic			Bolton Broth with Campylobacter Growth supplement, microaerophilic		
	Campylobacter spp. (log_{10}CFU/ml)	Background (log_{10}CFU/ml)	*Campylobacter* spp. (log_{10}CFU/ml)	Background (log_{10}CFU/ml)	Fluorescence[a]	*Campylobacter* spp. (log_{10}CFU/ml)	Background (log_{10}CFU/ml)	Fluorescence[b]
7S	9.15	-[c]	-[c]	6.08	- (919)	9.07	-[c]	+ (41244)‡
8S	-[c]	8.91	-	3.82	- (1244)	8.20	-	- (2516)
9S	-	8.96	-	6.94	- (816)	-[c]	9.39	+ (30581) †
10S	-	9.00	-	6.45	- (779)	-	8.95	+ (21156) †
11S	8.80*	-	-	3.21	- (1069)	-	8.41	+ (28360) ‡†
12S	-	8.90	-	7.32	- (974)	-	9.31	+ (21666) †
13S	9.40*	-	6.84*	-[c]	- (797)	5.50*	-	+ (11775)‡
14S	-/-[d]	5.45	-/-[d]	6.30	- (1178)	ND	ND	- (1046)
15S	-/-	2.83	6.45*	-	- (1052)	ND	ND	+ (10997)‡ †
16S	-/-	5.10	6.99*	-	- (942)	ND	ND	- (1410)
17S	6.50	-	-/-	8.19	- (1013)	-/-[d]	6.89	+ (24466)‡ †
18S	ND	ND	6.73*	-	- (831)	ND	ND	- (820)
19S	8.95*	-	6.76*	-	- (1680)	9.27*	-	+(48843)‡
20S	6.73*	-	ND	ND	- (1137)	8.99*	-	+(60711)‡
21S	7.10*	-	9.53*	-	- (1119)	8.28*	-	+(23527)‡
22S	6.86*	-	7.19*	-	- (919)	9.28*	-	+(21137)‡
23S	8.96*	-	-/-	6.53	- (1098)	9.09*	-	+(32698)‡
24S	8.97*	-	6.81*	-	- (1547)	8.66*	-	+(65000)‡

ND = no growth detected

a) + = fluorescence above detection limit of 1962; - = fluorescence below detection limit

b) + = fluorescence above detection limit of 3357; - = fluorescence below detection limit

c = no background flora or no suspect *Campylobacter* spp. colonies

d = confirmed to be non-*Campylobacter* spp. colonies

* = *C. jejuni*

† = positive with FISH, but no *Campylobacter* spp. found on plates from enrichment in BB-Sup.

‡ = positive with FISH, but *Campylobacter* spp. positive on plates from any enrichment

Table 4.4 Comparison of results from the FISH procedure with results of plating after enrichment in different media.

A. Aerobic enrichment in Bolton Broth with blood

	FISH	Plating
Positive	2	12
Negative	34	24

B. Microaerophilic enrichment Bolton Broth with *Campylobacter* Growth supplement.

	FISH	Plating
Positive	29	15
Negative	7	21

C. *Campylobacter* spp. present after enrichment in any of the three media

	FISH	Plating
Positive	29	23
Negative	7	13

5. Conclusion and Summary

The objective of the current study was to develop a procedure using Fluorescent *in situ* Hybridization (FISH) that would be capable of detecting *Campylobacter* spp. in naturally contaminated poultry samples. In addition to testing the FISH procedure, buoyant density centrifugation (BDC) as a purification method and several enrichment media were also examined.

The BDC procedure was evaluated for cell recovery, changes in cell concentrations, effect of centrifugation temperature and the ability to remove background particles that may interfere with FISH analysis. BDC did not significantly change cell counts before and after the procedure, but it did increase cell concentration by 0.6-1.4 $\log_{10}$ with a simple and rapid centrifugation step. Although, there appeared to be no significant differences between samples centrifuged at room temperature and refrigerated temperature, the lower temperature showed less variation in the results. In addition, BDC eliminated many particles from chicken homogenate samples which interfered with FISH processing. Overall, BDC was considered to be a simple and reliable procedure that could reduce inhibitory particles from samples containing a wide range of cell concentrations.

A FISH protocol that can be completed in microcentrifuge tubes was developed and evaluated for specificity with *C. jejuni* and non-*Campylobacter* isolates. The detection limit for pure culture *C. jejuni* was 7.33$\log_{10}$CFU/ml, and addition of an enrichment step prior to FISH was necessary. Several types of enrichment media were examined, including Bolton Broth with blood, Bolton Broth and TSB with *Campylobacter* Growth supplement and an aerobic semi-solid medium (Jeffery et al, 2000), all of which were incubated microaerophilically and aerobically. Bolton Broth with blood under both atmospheric conditions and microaerophilic Bolton Broth

with *Campylobacter* Growth supplement showed the best potential for supporting the growth of *C. jejuni*. Results of FISH after enrichment in each type of medium showed that the presence of blood can interfere with the processing of samples and significantly increases the background fluorescence. In order to eliminate this problem, a series of preparation steps were tested to be applied prior to the FISH procedure. The steps included lysing of the blood, BDC and a filtration through a 0.8μm membrane filter with the addition of a prefilter. After pre-treating the samples, background fluorescence was shown to be lower than seen with samples enriched in an blood-free medium.

The ability to detect *Campylobacter* spp. with FISH and plating in naturally contaminated samples was tested for both aerobic Bolton Broth with blood and microaerophilic Bolton Broth with *Campylobacter* Growth supplement. Microaerophilic Bolton Broth with blood was used as a "gold standard" to compare the results. Interestingly, Bolton Broth with *Campylobacter* Growth supplement was as effective as the current standard, suggesting that the blood-free medium could be used as a replacement. This would eliminate the need for pre-treatment steps prior to FISH, required to remove blood particles. Aerobic Bolton Broth with blood was only effective in culturing *Campylobacter* spp. in spiked, but not in naturally contaminated samples. The medium failed to produce as many positive samples as the other two media and the *Campylobacter* counts reached were also significantly lower. This may explain why the FISH procedure failed to produce positive results for this medium. Overall, there appeared to be a correlation between positive samples with FISH and those positive with conventional plating. However, there was a quite high percentage of false positives with the FISH procedure, suggesting that background bacteria may have either overgrown the *Campylobacter* spp. or resulted in non-specific binding of the probe.

In conclusion, the FISH procedure showed promise for the detection of *Campylobacter* spp. in poultry and positive results could be obtained in only 3 days. However, the method has areas that could be improved upon. Possible reasons for the rather high rate of false positives should be investigated. Also, the current FISH assay has a relatively high detection limit, which could possibly be reduced by altering the fluorescent label of the probe or by increasing the sensitivity of the equipment used to determine the amount of fluorescence. Overall, the FISH protocol developed would likely be the most effective using enrichment in blood-free Bolton Broth, which would eliminate several of the problems including background fluorescence and manageability of the blood cell debris. For samples containing no blood, such as live-bird swabs, this enrichment would best be followed directly by FISH. With samples containing blood residue, such as whole carcass rinse, the enrichment should be followed by lysis and BDC prior to FISH. The choice of probe appears to have been successful for the detection of not only *C. jejuni* but also other *Campylobacter* spp.

References

Achen, M., Morishita, T., and E. Ley. (1998). Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. Avian Diseases. **42**: 732-737.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and J. Watson. (1994). Molecular Biology of the Cell 3rd ed. New York: Taylor and Francis Group.

Altekruse, S., Stern, N., Fields, P. and D.L. Swerdlow. (1999). *Campylobacter jejuni* – An emerging foodborne pathogen. Emerging Infectious Diseases. **5**: 28-35.

Allos, B.M., Lippy, F.T., Carlsen, A., Washburn, R.G., and M. Blaser. (1998). *Campylobacter jejuni* strains from patients with Guillian Barré Syndrome. Emerging Infectious Diseases. **4**: 263-268.

Amann, R., Ludwig, W., and K.H. Schleifer. (1997). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology Reviews. **59**: 143-169.

Atanassova, V. and C. Ring. (1999). Prevalence of *Campylobacter* spp. in poultry and poultry meat in Germany. International Journal of Food Microbiology. **51**: 187-190.

Baylis, C., MacPhee, S., Martin, K., Humphrey, T., and R. Betts. (2000). Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. Journal of Applied Microbiology. **89**:884-891.

Berndtson, E., Tivemo, M., and A. Engall. (1992). Distribution and numbers of *Campylobacter* in newly slaughtered broiler chickens and hens. International Journal of Food Microbiology. **15**: 45-50.

Berrang, M., Smith, D., Windham, W., and P. Feldner. (2004). Effect of intestinal content contamination on broiler carcass *Campylobacter* counts. Journal of Food Protection. **67**: 235-238.

Blais, B and L. Phillippe. (1999). Growth of *Campylobacter jejuni* in various media under different atmospheric conditions. Journal of Rapid Methods and Automation in Microbiology. **7**: 291-300.

Chan, K., Tran, H.L., Kanenaka, R.Y., and S. Kathariou. (2001). Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at a low temperature (4°C). Applied and Environmental Microbiology. **67**: 4186-4191.

Chantarapanont, W., Berrang, M., and J. Frank. (2003). Direct microscopic observation and viability determination of *Campylobacter jejuni* on chicken skins. Journal of Food Protection. **66**: 2222-2230.

Chaveerach, P., ter Huurne, A., Lipman, L., and F. van Knapen. (2003). Survival and resurrection of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acidic conditions. Applied and Environmental Microbiology. **69**: 711-714.

Cook, N. (2003). The use of NASBA for the detection of microbial pathogens in food and environmental samples. Journal of Microbial Methods. **53**: 165-174.

Corry, J., and H. Atabay, (2001). Poultry as a source of *Campylobacter* and related organisms. Journal of Applied Microbiology. **90**: 96S-114S.

Coşkuner,G. (2002). A new molecular technique for the identification of micro-organisms in biological treatment plants: fluorescent in situ hybridization. Turkish Journal of Biology. **26**: 57-63.

Doyle, P., Beuchat, M. and A. Montville. (2001). Food Microbiology; Fundamentals and Frontiers 2nd ed. ASM Press, Washington, DC.

Engberg, J., On, S., Harrington, C. and P. Gerner-Smidt. (2000). Prevalence of *Campylobacter, Arcobacter, Helicobacter,* and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for *Campylobacters*. Journal of Clinical Microbiology. **38**: 286-291.

Ellerbroek, L. (1997). Airborne microflora in poultry slaughtering establishments. Food Microbiology. **14**: 527-531.

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

Fang, Q., Brockmann, S., Botzenhart, K., and A. Wiedmann. (2003). Improved detection of *Salmonella* spp. in foods by fluorescent in situ hybridization with 23 rRNA Probes: a comparison with conventional culture methods. Journal of Food Protection. **66**: 723-713.

Farkas J. (1998). Irradiation as a method for decontaminating food: a review. International Journal of Food Microbiology. **44**:189-204.

Glöckner, F., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K., and K. Schleifer. (1996). An in situ hybridization protocol for detection and identification of planktonic bacteria. Systematic and Applied Microbiology. **19**: 403-406.

Hahn, A. (1998). Guillian- Barré Syndrome. The Lancet. **352**: 635-641.

Halldórsdóttir, S., Englund, S., Nilsen, S.F. and I. Olsaker. (2002). Detection of *Mycobacterium avium* subspp. *paratuberculosis* by buoyant density centrifugation, sequence capture PCR and dot blot hybridization. Veterinary Medicine. **87**: 327-340.

Harris, D. (2002). Quantitative Chemical Analysis. 6th ed. W.H. Freeman and Company. New York, NY.

Hazeleger, W., Wouters, J., Rombouts, F., and T. Abee. (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperatures. Applied and Environmental Microbiology. **64**: 3917-3922.

Hunt, J., Abeyta, C., and T. Tran. (1998). Isolation of *Campylobacter* species from food and water. Bacteriological Analytical Manual 8th ed. AOAC International, Gaithersburg: 7.01-7.24. Available at www.cfsan.fda.gov/~ebam/bam-7.html, accessed on April, 2004

Jeffery, J., Hunter, A., and E. Atwill. (2000). A field suitable, semi-solid aerobic enrichment medium for isolation of *Campylobacter jejuni* in small numbers. Journal of Clinical Microbiology. **38**: 1668-1669.

Jørgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D., Bolton, F., Frost, J., Ward, L., and T. Humphrey. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. Internation Journal of Food Microbiology. **76**: 151-164.

Josefsen, M.H, Lübeck, P.S, Hansen, F. and J. Hoorfar. (2004). Towards and international standard for PCR-based detection of foodborne thermotolerant campylobacters: interaction of enrichment media and pre-PCR treatment on carcass rinse samples. Journal of Microbiological Methods. **58**: 39-48.

Ketley, J. (1997). Pathogenesis of enteric infection by *Campylobacter*. Microbiology. **143**: 5-21.

Lázaro, B., Cárcamo, J., Audícana, A., Perales, I., and A. Fernandez-Astorga. (1999). Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. Applied and Environmental Microbiology. **65**: 4677-4681.

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

Lindqvist, R. (1997). Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. International Journal of food Microbiology. **37**: 73-82.

Lindqvist, R., Norling, B. and S. Thisted Lambertz. (1997) A rapid sample preparation method for PCR detection of food pathogens based on buoyant density centrifugation. Letters in Applied Microbiology. **24**: 306-310.

Madigan, M., Martinko, J. and J. Parker. (2000). Brock Biology of Microorganism 9th ed. New Jersey: Prentice Hall.

Medeiros, D and L. Hofmann. (2002). Isolation of thermophilic *Campylobacter* from food. Compendium of Analytical Methods Vol.3. Health Products and Food Branch, Health Canada. MFLP-46. Available at http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_3/e_index.html, accessed on April, 2004.

Moreno, Y., Botella, S., Alonso, J., Ferrús, M., Hernandez, M., and J. Hernandez. (2003). Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. Applied and Environmental Microbiology. **69**: 1181-1186.

Moreno, Y., Hernandez, M., Ferrús, M., Alonso, J., S., Alonso, Montes, R., and J. Hernandez. (2001). Direct visualization of thermotolerant campylobacters in chicken products by PCR and in situ hybridization. Research in Microbiolgy. **152**: 577-582.

Moter, A., and U.B. Göbel. (2000). Fluorescence in situ hybridization for direct visualization of microorganisms. Journal of Microbiological Methods. **41**: 85-112.

Nachamkin, I., Allos, B.M., and T. Ho. (1998). *Campylobacter* species and Guillian Barré Syndrome. Clinical Microbiology Reviews. **11**: 555-567.

Nachamkin, I. and M. Blaser. (2000). Campylobacter, 2nd ed. ASM Press. Washington, DC.

Nachamkin, I. (2002). Chronic effects of *Campylobacter* infections. Microbes and Infection. **4**: 399-403.

Newell, G. and C. Fearnley. Sources of *Campylobacter* colonization in broiler chickens. Applied and Environmental Microbiology. **69**: 4343-4351.

Neumann, M. and D. Gabel. (2000). Simple method for reduction of autofluorescence in fluorescence microscopy. Journal of Histochemistry and Cytochemistry. **50**: 437-439.

Oxoid, Ltd. Bolton Selective Enrichment Broth. http://www.oxoid.com/uk/index.asp?mpage=iproductdetail&pre=CM0983&l=EN&x=campylobacterx, accessed on September, 2003.

Park, S. (2002). The physiology of *Campylobacter* species and its revelance to their role as foodborne pathogens. International Journal of Food Microbiology. **74**: 177-188.

Pearson, A. and T. Healing. (1992). The surveillance and control of campylobacter infection. Communicable Disease Review. **2**: R133-R138.

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

Pertoft, H. (2000). Fractionation of cells and subcellular particles with Percoll. Journal of Biochemical and Biophysical Methods. **44**: 1-30.

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

Rice, B., Lamichane, C., Joseph, S., and D. Rollins. (1996). Development of a rapid and specific Colony-lift Immunoassay for detection and enumeration of *Campylobacter jejuni, C. coli* and *C. lari*. Clinical and Diagnostic Laboratory Immunology. **3**: 669-677.

Sahin, O., Luo, N., Huang, S., and Q., Zhang. (2003). Effect of *Campylobacter*-specific maternal antibodies on *Campylobacter jejuni* in young chicks. Applied and Environmental Microbiology. **69**: 5372-5379.

Sahin, O., Zhang, Q., Meitzler, J., Harr, B., Morishita, T., and R. Mohan. (2001). Prevalence, antigenic specificity and bacterial activity of poultry anti-*Campylobacter* maternal antibodies. Applied and Environmental Microbiology. **67**: 3951-3957.

Sails, A., Fox, A., Bolton, F., Wareing, D., and D. Greenway. (2003). A real-time PCR assay for the detection of *Campylobacter jejuni* after an enrichment culture. Applied and Environmental Microbiology. **69**: 1383-1390.

Shreeve, J., Toszeghy, A., Ridley, A., and D. Newell. (2002). The carry-over of *Campylobacter* isolates between sequential poultry flocks. Avian Diseases. **46**: 378-385.

Slader, J., Domingue, G., Jørgensen, F., McApline, K., Owen, R.J., Bolton, F., and T. Humphrey. (2002). Impact of transport crate reuse and of catching and processing on *Campylobacter* and *Salmonella* contamination of broiler chickens. Applied and Environmental Microbiology. **68**: 713-719.

Stern, N. and C. Robach. (2001). Enumeration of *Campylobacter jejuni* in broiler feces and corresponding processed carcasses. Journal of Food Protection. **66**: 1557-1563.

Stern, N., Fedorka-Cray, P., Bailey, J., Cox, N., Craven, S., Hiett, K., Musgrove, M., Ladely, S., Cosby, D., and G. Mead. (2003). Distribution of *Campylobacter* spp. in selected US poultry production and processing operations. Journal of Food Protection. **64**: 1705-1710.

Suresh, P., and J. Rehg. (1996). Comparative evaluation of several techniques for purificationn of *Cryptosporiduim parvum* oocytes from rat feces. Journal of Clinical Microbiology. **34**: 38-40

Tholozan, J., Cappelier, J., Tissier, J., Delattre, G. and M. Federigh. (1999). Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. Applied and Environmental Microbiology. **65**: 1110-1116.

USDA. Nationwide Broiler Chicken Microbiological Baseline Data Collection Program. (1995) www.fsis.usda.gov/OPHS/baseline/contents.htm, accessed on May, 2004.

Uyttendaele, M., Schukkink, R., Gemen, V. and J. Debevere. (1995). Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). Applied and Environmental Microbiology. **64**: 1341-1347.

Uyttendaele, M., Debevere, J. and R. Lindqvist. (1999). Evaluation of buoyant density centrifugation as a sample preparation method for NASBA-ELGA detection of *Campylobacter jejuni* in foods. Food Microbiology. **16**: 575-582.

van de Giessen, A., Mazurier, S., Jacobs-Reitsma, W., Jansen, W., Berkers, P., Ritmeester, W., and K. Wernars. (1992). Study on the epidemiology and control of *Campylobacter jejuni* in poultry broiler flocks. Applied and Environmental Microbiology. **58**: 1913-1917.

Van Horne, K. and C. Tóth. (1999). Evaluation of the AnaeroPack Campylo System for growth of microaerophilic bacteria. Journal of Clinical Microbiology. **37**: 2376-2377.

Wagge, A., Vardund, T., Lund, V., and G. Kapperud. (1999). Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a semi-nested PCR assay. Applied and Environmental Microbiology. **65**: 1636-1643.

Wang, H., Farber, J.M., Malik, N., and Sanders, G. (1999). Improved PCR detection of *Campylobacter jejuni* from chicken rinses by a simple sample preparation procedure. International Journal of Food Microbiology. **52**: 39-45.

Wassenaar, T., and M. Blaser. (1999). Pathophysiology of *Campylobacter jejuni* infections of humans. Microbes and Infection. **1**: 1023-1033.

Wedderkopp, A., Gradel, K., Jørgensen, F., and M. Madsen. (2001). Pre-harvest surveillance of *Campylobacter* and *Salmonella* in Danish broiler flocks: a 2-year study. International Journal of Food Microbiology. **68**: 53-59.

Winters, D., O'Leary, A. and M. Slavik. (1997). Rapid PCR with nested primers for direct detection of *Campylobacter jejuni* in chicken washes. Molecular and Cellular Probes. **11**: 267-271.

Woldringh, C., Binnets, J. and A. Manns. (1981). Variation in *Escherichia coli* buoyant density measured in Percoll gradients. Journal of Bacteriology. **148**: 58-63.

Xue, Q., Renault, T., Conchennec, N., and A. Gerard. (2000). Separation of European flat oyster, *Ostrea edulis*, haemocytes by density gradient centrifugation and SDS-PAGE characterization of separated haemocyte sub-populations. Fish and Shellfish Immunology. **10**:155-156.