

**Differentiation between Quinolone Resistant and Sensitive
Isolates of *Campylobacter jejuni* by a Multiplex PCR Assay**

By

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I certify the content of the thesis to be my own and original work and that all sources have been accurately reported and acknowledged by complete references, and that this document has not previously been submitted in its entirety or in part at any educational establishment.



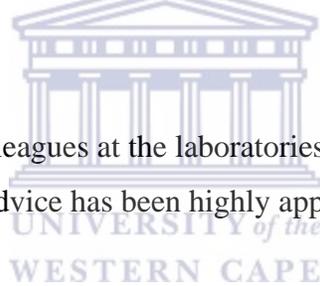
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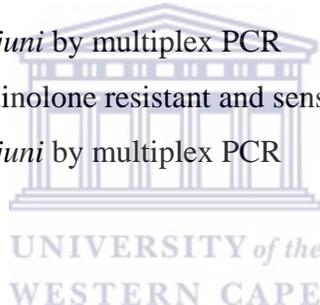
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LIST OF ABBREVIATIONS

PCR	Polymerase chain reaction
mm	millimeter
O ₂	Oxygen
CO ₂	Carbon Dioxide
°C	degrees celsius
NaCl	Sodium chloride
<	less than
>	greater than
ca.	approximately
Gyr A	Gyrase A
et al.	and others
Thr	Threonine
Asp	Asparagine
Ala	Alanine
QRDR	Quinolone resistance determining region
um	micrometer
ul	microliter
NCCLS	National Committee for clinical laboratory standards
TE	Tris, EDTA buffer
EDTA	Ethylenediamine tetra-acetic acid
DNA	Deoxyribonucleic acid
bp	base pairs
Ile	Isoleucine
ss	single stranded
ug	micrograms
oligo	oligonucleotides
DNTP's	Deoxyribonucleotide triphosphates
MgCl ₂	Magnesium chloride

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CHAPTER 1

INTRODUCTION

For the past two decades *Campylobacter* species have been implicated in many food-borne illnesses across the world (Friedman *et al.* 2000). Their incidences have far outnumbered those cases of *Salmonella* and other foodborne organisms collectively (Allos, 2001). *Campylobacter jejuni*, one of eighteen species described in the genus *Campylobacter* to date have frequently been reported (since 1992 by Tauxe *et al.*) as the most common cause of disease in this genus. *Campylobacter jejuni* has therefore become the primary focus worldwide for many researchers in many Food Research Laboratories.

Over the years, some information has been gathered concerning the epidemiology and pathogenicity of *C. jejuni* (Anderson *et al.* 2001; Kramer *et al.* 2000). With this information, researchers were able to predict why and how outbreaks or even sporadic cases of the disease occurred. *Campylobacterioses*, being a zoonotic disease has placed great strain upon the shoulders of the food industry in general and as such has called for the demand for knowledge on how to control and eliminate these pathogens entirely (Vacher *et al.* 2003; Engberg *et al.* 2001).

Today the world faces a new challenge and this is “Antimicrobial Resistance”. Resistant organisms are more pathogenic, harder to kill and hence more of a threat to the immunocompromised. They prolong diseases due to the fact that antibiotics in use may prove to be useless, thereby stretching hospital stays and ultimately hospital costs. Aggravation of the disease through development into secondary infections can sometimes

even cause death (Engberg *et al.* 2004; Nelson *et al.* 2004). *Campylobacterioses* are generally self-limiting and does not usually require the use of antimicrobials (Blaser *et al.* 1995; Engberg *et al.* 2001). However, antibiotics are prescribed in severe cases of the disease, to prevent any secondary complications such as bacteremia or meningitis (Skirrow *et al.* 1995). It is usually through the misuse of these antibiotics that antibiotic resistant organisms develop. Over-prescribing antibiotics for conditions like the flu or common cold that cannot be treated with these medicines also contribute to antibiotic resistance. Probably more than fifty percent of the antibiotics prescribed by doctors are unnecessary (Sustainable Table: The issues: Antibiotics, 2005. [Online]. Available www.sustainabletable.org/issues/antibiotics/).

Apart from human abuse and misuse of antibiotics, the prudent use of antimicrobials in animal feeds and in agriculture has further engendered the development of resistant pathogens (Smith *et al.* 1999; Piddock, 1998; Sam *et al.* 1999). These pathogens are passed along in the food chain to humans, where they cause serious complications and infections. Antibiotics are fed unnecessarily to livestock, poultry and fish to promote slightly faster growth and to compensate for the unsanitary conditions often found on factory farms. Modern factory farms where thousands of animals are crowded into small areas are heavily dependent upon the use of antibiotics. These animals live in stressed, unnatural conditions thus being prone to diseases and death. To aid survival, these industrial operations probably use antibiotics generously (Sustainable Table: The Issues: Antibiotics, 2005 [Online]. Available <http://www.sustainabletable.org/issues/antibiotics/>). One classic example is the use of quinolones and fluoroquinolones in animal feeds to enhance the growth rate of food animals and chickens (Velazquez *et al.* 1995). Quinolones used to be the drug of choice for treatment of gastroenteritis caused by *Campylobacter* but since its

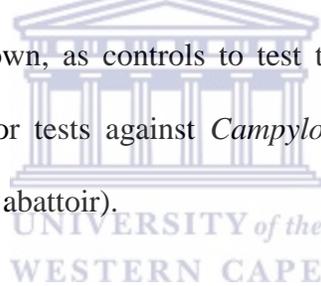
introduction into animal feeds (in 1995 in developed countries), whether through legislation or not, doctors have lost all hope in prescribing quinolones where necessary (Smith et al. 1999; Piddock LJV, 1998; Sam et al. 1999). Instead the goal here is to increase efficiency through speeding up growth. By enhancing growth rate, farmers and food producers are not only able to meet the demands of the consumers but to also benefit themselves through expanding their income and profit margins.

A first step in controlling or eliminating the usage of antibiotics in animals, especially in South Africa, would be to put proper procedures in place and make documentation available. With information available, suggestions can be made on how to target or overcome the resistance problem. Right now there is not much to go on due to the lack of involvement by government. The Department of Health and the Department of Agriculture which are currently separate bodies should work together as a single entity with a common goal in mind. And these are to produce safe foods that are free of any form of contamination.

Scientists, however, are working hard to find new ways to defeat bacteria that are increasingly resisting the antibiotics already available. One approach is to work against the very characteristics that make a bacterium resistant. That is to adapt the antibiotic so that it recognises the mutant bacteria and destroy them before they reproduce. Another approach is to involve exploiting bacteria's natural enemy- viruses known as bacteriophages, which enter the bacterium and kill them. They have proven more effective than antibiotics in some animal studies. However, bacteriophages are extremely specific and have to be matched to exact strains of bacteria. Recently though the development of newer fluoroquinolones have given some hope to potentially limit the emergence of

fluoroquinolone resistance by possessing a dual-binding mechanism of on both the DNA gyrase and Topoisomerase IV (the targets of fluoroquinolones). Owing to the rarity of double mutations occurring within both these enzymes [simultaneously, the newer fluoroquinolones may just be strong enough to put a halt on the rapid emergence of fluoroquinolone resistance \(Fisher and Heaton, 2003; Smith *et al.* 2002; Bachoual *et al.* 2001; Luo *et al.* 2003; Payot *et al.* 2002\).](#)

The objectives of this study were therefore to design a polymerase chain reaction (PCR) procedure from the database in genbank for *Campylobacter jejuni* for the differentiation between quinolone sensitive and resistant isolates. This was followed by using pre-determined quinolone antibiotypes of clinical *Campylobacter jejuni*, from the Red Cross Children's Hospital in Cape Town, as controls to test the PCR procedures against, and thereby determine a standard for tests against *Campylobacter jejuni* isolates other than clinical samples (eg. poultry and abattoir).



In South Africa, the use of antibiotics in animal feeds may still be in its prime, and people may not even be aware of it, but the evidence is there. Fluoroquinolone resistant *C. jejuni* isolates were isolated from human and poultry subjects and the fact that they are present in chickens suggest that they are transmitted from the farm environment somehow.

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CHAPTER 2

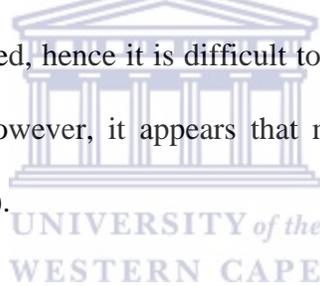
LITERATURE REVIEW

2. *Campylobacter*

Before 1963 *Campylobacter* was classified in the genus *Vibrio* when Sebald and Veron proposed the genus name *Campylobacter*. *Campylobacter* currently consist of about eighteen species, some of which are further divided into sub-species. Some members of this group have been associated with disease in animals for many years. However, their role in human disease has been known for only the past 20- 25 years. In many countries, *Campylobacters* are the most frequently isolated organisms in patients suffering gastroenteritis, more so than *Salmonellae* (Allos, 2001; Chan *et al.* 2003; Allos and Blaser, 1995). *C. jejuni* is first in line causing most such illness, followed by *C. coli* (Grifiths *et al.*, 1990; Nachamkin, 1999; Friedman *et al.* 2000). *C.coli* causes about 3-5% of human cases of *Campylobacteriosis*, although the extent of its implications can be higher in some areas (Martin *et al.* 1988; Popovic-Uroic, 1989). Others such as *C. upsaliensis* and *C.laridis* have also been isolated from human stools. As *C. jejuni* appears to be the most important isolate associated with disease caused by *Campylobacters*, as cited in the literature, it was researched in detail as a basis for this study (Engberg *et al.* 2004; Kramer *et al.* 2000; van Diest and de Jong, 1999; Zirnstein *et al.* 1999; Tauxe, 1992; Taylor, 1992).

2.1 *Campylobacter jejuni*

Campylobacter jejuni is now recognised as the leading cause of bacterial diarrheal illnesses in the United States causing more disease than *Shigella* and *Salmonella* species combined (Zirnstein et al. 1999). Before 1972, when methods were developed for its isolation from faeces, it was believed to be primarily an animal pathogen causing abortion and enteritis in sheep and cattle. Although commonly found in healthy cattle, chickens and flies, it has also been isolated in wombat and kangaroo faeces being the cause of bushwalker's diarrhoea (Norcross et al. 1992). It naturally colonises many different bird species. Pathogenic mechanisms are still being studied, hence it is difficult to differentiate between pathogenic and non-pathogenic strains. However, it appears that many of the chicken isolates are pathogens (Andersen et al. 2005).



2.1.1 Identification

Colonies are non-haemolytic and may be flat, spreading and with an irregular edge or a discrete, circular-convex shape with a diameter of 1-2 mm. Suspect colonies are examined microscopically for the characteristic gram negative, slender, curved and motile rod. They are microaerophilic with an atmospheric preference of 5% O₂ and 10% CO₂, grows best at 42 °C and is very sensitive to heat and drying. Isolates are tested for catalase, oxidase, nitrate reduction, hippurate hydrolysis and sensitivities to various antibiotics (Penner, 1988; Anderson et al. 2001; Ishihara et al. 2004).

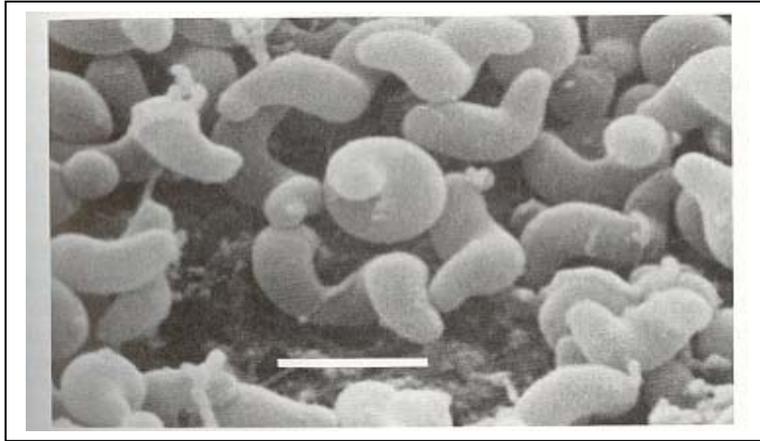


Figure 2.1: Scanning electron micrograph of *C. jejuni* illustrating the curved shape of most cells and the coccus shape of some (Taken from Fundamentals of Microbiology, by Alcamo IE, 4th edition, 1994)

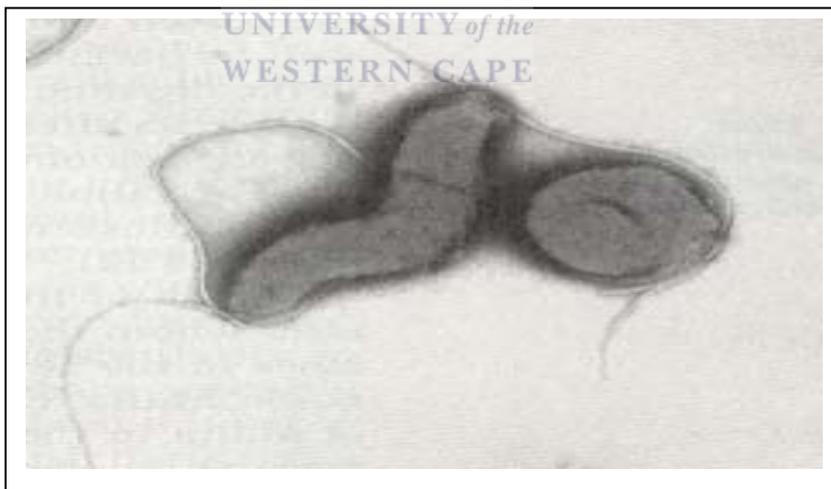
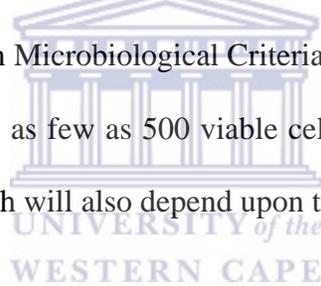


Figure 2.2: Transmission electron micrograph of negatively stained cells showing flagellation of both types of cells (Taken from Fundamentals of Microbiology, by Alcamo IE, 4th edition, 1994).

2.1.2 Distribution and Associated Foods

Campylobacter jejuni can be harboured in the intestines of a large variety of wild and domestic animals, with no evidence of illness in them (Franco, 1989; Skirrow, 1991; Kramer *et al.* 2000). Specifically, *C.jejuni* has been isolated from cattle, chickens, birds, flies, raw or semi-pasteurised milk, and unchlorinated waters such as ponds and streams (Anderson *et al.* 2001). The organism can infect man after direct contact with animals, or indirectly via contaminated water, milk, meat, or faecal contact during processing of meat (Kramer *et al.* 2000; Aarestrup *et al.* 1997; Norcross *et al.* 1992). As mentioned by the National Advisory Committee on Microbiological Criteria for foods, in 1993 in the Journal of Food Protection 57, ingesting as few as 500 viable cells are sufficient to cause disease. The severity of the disease though will also depend upon the dose (Anderson *et al.* 2001).



The bacterial cells can survive in surface waters for up to 4 weeks at 4 °C (Blaser *et al.* 1980). Municipal waters can cause disease if not treated properly. Human enteritis occurs frequently in rural areas and hikers drinking on natural waters (Blaser *et al.* 1984; Hopkins *et al.* 1984). Raw milk is usually contaminated from infected udders, but the milk can also be contaminated via animal faeces (Skirrow *et al.* 1995; Hudson *et al.* 1984; Hutchinson *et al.* 1985; Morgan *et al.* 1985). Raw or undercooked poultry are usually the biggest cause of human infection (Jorgensen *et al.* 2002; Dufrenne *et al.* 2001; Blaser *et al.* 1984). *Campylobacter* occurs less frequently on red meats, but they are present (Doyle, 1984). They flourish in offal meats (Bolton *et al.* 1985; Fricker and Park, 1989). Pets eat this, thereby passing it onto humans. Since *C.jejuni* cannot grow at temperatures below 30 °C,

growth does not usually occur in foods (ICMSF,1996). Conditions affecting survival are more important, since a sufficient number of cells must survive to form an infective dose. The growth of cells is enhanced by blood or fluids obtained from thawing. These factors are responsible for contaminating work surfaces and hands of food handlers, thereby causing self-infection. The handling and preparation of raw meats in domestic kitchens presents a great risk of direct hand-to-mouth exposure to enteropathogens and cross-contamination of the kitchen environment with other ready to eat foods (Kramer *et al.* 2000; Coates *et al.* 1987). A study done by Moore and Madden in 1998 have shown the microbial status of the livers of food animals to be a good indicator of slaughterhouse hygiene practices. The cause of contamination within the liver arises from ruptured intestinal contents during evisceration. *C. jejuni* has been reported to form viable-but-non-culturable (VNC) forms when subjected to stress (Jones *et al.* 1991) and there are claims that VNC's can colonize the intestinal tract of chickens (Pearson *et al.* 1993). However, other researchers have been unable to demonstrate such infection (Medema *et al.* 1992; Beumer *et al.* 1992), and the role of VNC's in transmission of *Campylobacter* remains uncertain.

2.1.3 Isolation Procedures and Recovery from Foods

Campylobacter jejuni is present in high numbers in stool samples and can be isolated through direct plating onto special antibiotic-containing media. Since foods may contain only a few cells, or cells that have been injured by food processing and preservation procedures, pre-enrichment in liquids are normally required to successfully detect or determine microbial contamination of food (Post, 1995). Bolton Selective Enrichment Broth which is intended for the pre-enrichment of *Campylobacter* organisms in food

samples increases the number of cells available for culture, first by resuscitating injured organisms and then encouraging them to multiply (Bolton, 1995). *Campylobacter* species grows well microaerophilically at 42 °C (10% CO₂; 5% O₂) for 48 hours. However, the presence of toxic products of oxygen such as hydrogen peroxide and superoxide anions prevents growth of certain cells. Other cells may be damaged by chilling, heating, and freezing. These lethally and sublethally damaged cells may be revived by pre-enrichment in certain selective or non-selective broths at 37 °C for 4 hours prior to incubation at 42 °C (Humphrey 1986, 1989). Various enrichment broths are available containing various antimicrobials thereby preventing contamination of other bacteria and selecting for *Campylobacter*. However, researchers investigating *Campylobacter* contamination of raw meat and poultry have used a Preston *Campylobacter* Selective Enrichment Broth (Oxoid) for pre-enrichment followed by sub-culturing onto modified charcoal cefoperazone desoxycholate agar medium and the results showed a preferential selection for *C. coli* rather than *C. jejuni*. This therefore emphasizes the significance of an appropriate choice of isolation regimes and the value of combining epidemiological typing techniques when investigating human pathogens in the food chain (Kramer *et al.* 2000; Ishihara *et al.* 2004). Enrichment cultures shaken can be plated after 24 hours whereas stationary cultures can be streaked after 48 hours. Enumerations in foods are done by the Most Probable Number (MPN) technique or the direct plating technique. The use of membrane filtration combined with membrane overlay on selective or non-selective media has proved useful when isolating *Campylobacter* species from foods (Baggerman and Koster, 1992).

2.1.4 Survival Characteristics

Generally, *C. jejuni* has rather simple growth requirements. However, their ability to survive and proliferate will depend upon certain upper and lower limits for growth, as depicted by the table below:

Table 2.1: Limits for Growth of *C.jejuni*

	Minimum	Optimum	Maximum
Temperature	32	42-43	45
pH	4.9	6.5-7.5	Ca.9
NaCl (%)	0	0.5	1.5
Water activity	>0.987	0.997	–
Atmosphere*	–	5% O ₂ +10% CO ₂	–

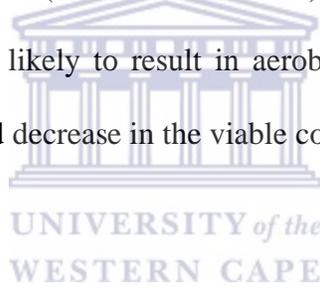
*Aerotolerance is enhanced by compounds quenching toxic derivatives of oxygen; oxygen required for growth

2.1.4.1 Survival Under Frozen Conditions

A decline in the viable count of *C. jejuni* has been observed within the first day of freezing. This decline drops after a certain time. Survival of cells is usually influenced by food types and pH of food (Barrell, 1984). For example, usually more cells are observed in frozen meat rather than in skim milk.

2.1.4.2 Survival and growth from -1°C to 47°C

Campylobacter jejuni grows best at 42°C, but the growth rate occurs between 32°C – 45°C. Anything above or below this range promotes death of cells. They survive better in chilled than in frozen foods (Hood *et al.* 1988). Their death rate is greater in raw milk than skim milk due to the presence of lactoperoxidase. They do not survive well in egg white, frozen raw beef cubes and in the interior and under the crust of cooked meat pies. This is due to the lack of oxygen in the latter two food types and the presence of conalbumin in the high pH of egg white. Their survival rate, however, is better in egg yolk, liquid egg and chilled foods rather than in milk or water (Beumer *et al.* 1985). In general, storage of foods at chilled temperatures (0-5 °C) is likely to result in aerobic spoilage of moist fresh meats before more than about a ten fold decrease in the viable count of contaminating *C.jejuni* has occurred.



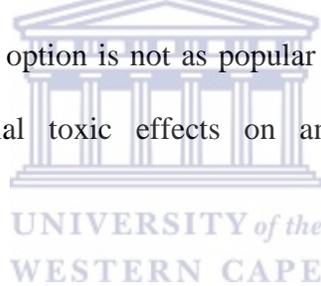
2.1.4.3 Heat

Campylobacter jejuni is readily destroyed by heat. Different strains offer different heat sensitivities. The greatest heat resistance is inferred when the pH of food is closer to 7 or neutrality. Therefore, resistance will decrease as the pH moves above or below pH 7 (Gill and Harris, 1982). Ground meats and fresh intact pieces of meats propose different challenges to heat exposures. Ground meats normally includes small cuts and trimmings, thus an organism on the carcass or cut surface may be ground into the meat. This ultimately distributes organisms into the center of the product. Hence, cooking of ground meat would need a specific temperature to kill any foodborne organisms located in the

center of a portion of meat. In contrast, the intact cuts of meat would be expected to contain foodborne pathogens on the external surfaces of the meat where the high temperatures of cooking would normally contact and kill the organisms (Anderson *et al.* 2001).

2.1.4.4 Irradiation

Campylobacter jejuni is sensitive to both ultra-violet (UV) and gamma-radiation (more sensitive to UV than *Escherichia coli* and more sensitive to gamma-radiation than *Salmonellae*. Sensitivity to irradiation is not necessarily affected by the pH of foods (Butler *et al.* 1987). Irradiation of food sources to destroy *C. jejuni* cells has been considered as an option but this option is not as popular because of public belief that this procedure may render potential toxic effects on an individual (Forth Worth-Star telegram,1998).



2.1.4.5 Water Activity

The optimal salt content for *C. jejuni* in brucella broth at 42 °C is 0.5%. In the absence of salt, the lag phase is extended. The sensitivity to salt indicates that *C. jejuni* will not grow in beverages or high-moisture foods with a high salt content. It has been observed that as storage temperature decreases, cells survive longer at comparable salt concentrations. Therefore a salt concentration of 2.5% in ground cod has little effect on the survival of *C. jejuni* at 6°C but the death rate increases when temperature is increased to 10°C. This is due to the production of peroxides because of lipid auto-oxidation catalyzed by salt. In processed foods where moisture is moderate, the inhibitory level of salt is less than 2% (Abraham and Potter, 1984).

Campylobacter are very sensitive to drying. The reduction in viability due to drying is dependent on the suspending medium, temperature and extent of drying. Surfaces contaminated with *C. jejuni* in liquids show a decrease in cells after drying. Conversely, due to *Campylobacter's* need for a low oxygen environment (<5%) and their susceptibility to drying, do the open surfaces of carcasses, meat, processing equipment and household surfaces limit their survival to a certain extent (Anderson *et al.* 2001). Blood or chicken thawing liquid influences the survival of cells. The blood and other tissue fluids provide a protection mechanism for cells, and if in high numbers can result in cross-contamination or auto-infection. Pig, sheep and cattle carcasses are air-dried. The temperature difference between the carcass surface (ca 30 °C) and the cold air (ca 0.5 °C) results in moisture evaporating into the cooling air-stream, thereby drying the carcass surface. Poultry are usually chilled in ice-cold water, precluding drying, before being packaged / stored in high moisture conditions after chilling. Offal meats from red meat carcasses often do not undergo significant drying (Abraham and Potter, 1984).

2.1.4.5 pH

The optimum pH range for *C. jejuni* is about 6.5 – 7.5 with a maximum of 9.0 – 9.5. The pH change in certain foods may not significantly alter the survival rate of cells. In raw milk or semi-pasteurised milk where the lacto-peroxidase system is not destroyed; increasing the pH to about 7.5 results in a lower activity of the system and hence a support for *C. jejuni* growth, whereas lowering the pH to 5.5 results in increasing its activity thereby promoting death of *C. jejuni*.

The minimum pH allowing growth is affected by the acidulant. The acidulant also determines the survival of cells. For example, in yoghurt a rapid increase in the death rate where growth is not permitted is due not only to the pH but also the organic acids produced by the fermentation of lactose. The death rate increases with an increase in temperature and lowering of the pH (Stern et al. 1985).

C. jejuni dies rapidly at low pH (below pH 4), especially at non-refrigerant temperatures. Hot acetic acids (0.25 – 0.5%) dips are used for chicken carcasses to reduce the load of *Campylobacter*. Experiments with 0.5% acetic acid at 50 °C for 90s used as a dip resulted in a six fold decrease of *Campylobacter* cells (Doyle and Roman, 1981).

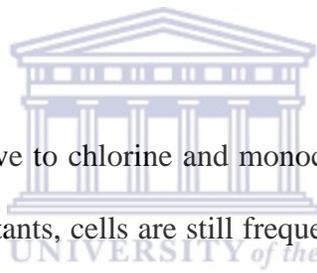
2.1.4.6 Food Ingredients and Additives

Sodium bisulphite in brucella broth protects *C. jejuni* against death. Bisulphite presumably protects cells from the harmful effects of oxygen and its derivatives. Hence sodium bisulphite can be used in milk to protect cells. Milk that undergoes low temperature pasteurisation allows its lactoperoxidase system to survive. This system kills off *C. jejuni* to a small extent due to the accumulation of toxic oxygen species. A higher pasteurisation temperature at 85°C for 10s causes inactivation of the lactoperoxidase system. The addition of normal levels of lactoperoxidase to milk that has undergone more severe heat treatments can further promote the rapid death of *C.jejuni*. In nutrient broth, ascorbic acid deteriorates into oxygen free radicals causing death to cells. Iso-ascorbate seems to auto-oxidise at a lower rate which is converse to sterile meat products tested (Beumer *et al.* 1985; Rhodehamel and Pearson, 1990).

2.1.4.7 Gases

The presence of oxygen can result in the death of many *Campylobacter* cells in milk and brucella broth. However, on raw meat, the presence of oxygen may have little effect on the survival of *Campylobacter* especially the initial air surrounding the meat. Upon packaging food, the packaging material may restrict gaseous exchange and the metabolic activity of food, thereby resulting in an atmosphere different from that of air. Certain activity of food such as that of catalase and superoxide dismutase gets rid of oxygen free radicals and allows survival and growth of present contaminating *Campylobacter* cells (Beuchat,1987).

2.1.4.8 Disinfectants



Campylobacters are very sensitive to chlorine and monochloramine. However, in spite of their sensitivity to these disinfectants, cells are still frequently found on poultry meats after chilling in chlorinated water. The numbers are however highly reduced. Reduction of cells could also very possibly be as a result of physical removal. Presumably, many cells are protected against chlorine within the surface tissues. *Campylobacter* exposed to chlorine are sublethally damaged and about 38-95% of these damaged cells may not grow on Skirrow's agar but can still grow on blood agar. Sublethally damaged cells are often unable to form colony growth on selective media (Blaser et al. 1986).

2.1.4.9 Control Mechanisms

Water can be contaminated by sewerage outfalls, farm run-offs, wild and domesticated farm animals (Anderson *et al.* 2001). Untreated ground and surface waters is a common

source of infection as well as water distribution systems resulting in sporadic cases of disease. *Campylobacters* are atleast as sensitive to chlorine and monochloramine as *E. coli*. Hence, treatment of water with chlorine together with maintenance of water distribution systems can prevent infection (Wang *et al.* 1983; Blaser *et al.* 1986).

Raw milk may be contaminated with *C. jejuni* by udder infection or during milking from animal faeces (Aarestrup *et al.* 1997). Control points and prevention can be made possible by washing and cleaning of udders, washing of hands and equipment used during milking but complete protection is almost impossible. There may be a low grade mastitis which may go unnoticed (Hudson *et al.* 1984). Raw milk consumption in certain countries is allowed as their safety is determined by the total viable count of organisms present. However, these tests do not prove the milk safe in its entirety. Raw milk chilled may prevent growth of other organisms, but *C. jejuni* is still able to survive and multiply. Pasteurisation can kill off *Campylobacters* and is the only control point in preventing infection from milk (Anderson *et al.* 2001).

Offal's of poultry and red meats are frequently contaminated with *C. jejuni* and *C. coli* at retail sales (Jorgensen *et al.* 2002; Dufrenne *et al.* 2001). Contamination of offal surfaces usually occur during surface evisceration and dressing. Taking care during these processes as well as physical removal of offal from intestines allows control. The offal carrying *Campylobacter* can be cooked at 55 °C – 60 °C for several minutes thereby causing death of cells. Cross-contamination of ready to eat foods, hands of workers, and kitchen equipment occurs from surfaces where raw offal have been placed. Offal's are washed at abattoirs, but this is not sufficient to reduce the bacterial load. Often too, offal's are chilled but not dried

on its surfaces; thereby allowing growth of *Campylobacter* (Bolton *et al.* 1985; Fricker and Park, 1989).

Many epidemiological studies reveal a connection between handling and consumption of poultry and human infection (Friedman *et al.* 2004; Neimann *et al.* 2003; Kapperud *et al.* 2003; Effler *et al.* 2001; Studahl and Anderson, 2000; Neal and Slack, 1997). Chicken carcasses become contaminated during de-feathering and evisceration and are still found contaminated at retail sales (Oosterom *et al.* 1983; Hood *et al.* 1988). Although poultry is pretreated at the abattoirs with chlorine and chilling, this is still not significant to allow complete removal of viable cells. Vertical infection between chickens in a flock does not occur. Chicks often become infected from extraneous sources such as contaminated water, litter, feed, wild birds, rodents, boots of workers and other farm flora (Shanker *et al.* 1990). A control point would mean complete removal of infected chicks from a flock. A chicken can be completely free of *Campylobacters* and if placed into a house where prior infection occurred three weeks or more ago, it would remain healthy, provided, extraneous sources are controlled. The caeca microbiota renders ineffective against *Campylobacters*, hence there is no protection there.

Risk factors in decreasing significance are: feeding broilers undisinfected waters; tending other poultry prior to entering the broiler house; tending to pigs prior; and geographic region and season (Kapperud *et al.* 1993).

There are two ways in which *C. jejuni* on raw meats can remain on ready to eat foods and cause infection: either by eating raw or undercooked meat or by cross-contamination of cooked foods.

Campylobacter jejuni is readily killed by cooking temperature between 55-60 °C for several minutes. However it is the handling of food after it has been cooked that is important (Coates *et al.* 1987).

2.2 *Campylobacteriosis*

Since the early 1970's, *Campylobacteriosis* has emerged from being an obscure disease in animals to recognition as a widespread intestinal disease in humans. *Campylobacteriosis* is the name of the illness caused by *C. jejuni*. It is also often known as *Campylobacter* enteritis or gastroenteritis (Engberg *et al.* 2004). Food poisoning caused by *Campylobacter* species can be severely debilitating but is rarely life-threatening. However, it has been linked with subsequent development of the neurodegenerative disease Guillian-Barre syndrome (GBS) (Wassenaar *et al.* 2000). Wassenaar and colleagues have reported that atleast thirty percent of GBS patients have had *C. jejuni* infections prior to the onset of neurological symptoms, and that the isolate most responsible for that in the Cape Town area are the 0:41 serotypes rather than the 0:19 serotypes, which are found at a frequency three times higher than the 0:41 counterparts (Wassenaar *et al.* 2000).

2.2.1 Symptoms

Infection by *C. jejuni* results in abdominal pain, fever, headaches, nausea vomiting, and ofcourse diarrhoea, which may be either sticky or watery and contain blood or leukocytes. Watery diarrhoea is very common in developing countries. Faeces that contain blood, mucus or leukocytes are referred to as the dysentery like syndrome (Allos and Blaser, 1995).

The disease starts to set in after 2-5 days after ingestion of contaminated food or liquids. It lasts for as long as 7-10 days and usually is self-limiting. Sometimes a person may be infected and still show no signs of illness, therefore host susceptibility may dictate the infectious dose to some degree. The type of diarrhoea may also dictate the pathogenicity of the organism. Upon reaching the small intestine, the organism may either adhere to the small intestine surface or cause watery diarrhoea; or it may invade the mucosal epithelial cells and cause bloody diarrhoea and colo-rectal inflammation (Allos and Blaser, 1995). *Campylobacter* from any source produces a heat labile cytotoxic enterotoxin which is similar to the LT B subunit enterotoxin of *E. coli*. Easy penetration of the mucosa is attributed to the rapid motility and shape of the organism (Anderson *et al.* 2001).

2.2.2 Diagnosis

A disease can only be diagnosed once an individual shows symptoms ascribed to a particular disease. For example, an individual may still appear healthy and display no signs of infection, yet *C. jejuni* can be found in the stools (Anderson *et al.* 2001). The disease is diagnosed after culturing the organism on a suitable growth medium, followed by further biochemical tests to aid and confirm identification.

2.2.3 Frequency of Disease

Illnesses caused by *Campylobacter jejuni* is currently the leading cause of disease in the United States. Approximately 2-4 million cases are reported annually, much higher than the estimated cases of Salmonellosis (Zirnstein *et al.* 1999). In other countries, such as the United Kingdom, Denmark and Sweden, *Campylobacter* is reported in the literature as the

most frequently isolated organisms in human disease (Andersen *et al.* 2005; Friedman *et al.* 2000; Neiman *et al.* 1998). Unfortunately, in South Africa there is no formal reporting system available and as such much information about isolated incidences and sporadic cases of the disease are lost.

Campylobacteriosis occurs more frequently in summer than in winter and amongst children and adult males. It is estimated that 500 persons may die from *Campylobacter* infections each year in the United States (Anderson *et al.* 2001).

2.2.4 Complications of Disease

Complications are rare, but they can occur. Conditions such as reactive arthritis, hemolytic uremic syndrome and septicemia followed by infections of nearly any organ have been reported (Blaser *et al.* 1995; Skirrow *et al.* 1995). Other cases of severity are meningitis, recurrent colitis and Gillian-Barre Syndrome (Wassenaar *et al.*, 2000). This syndrome occurs when the body's immune system is triggered to attack the body's own nerve cells, which may result in temporary or permanent paralysis. The reported incidence of fatalities in the United States is about 1 in every 1000 cases of *C. jejuni* infections (Blaser 1997).

2.2.5 Treatment

There is no specific treatment for this disease, but the objective is to drink plenty of fluids to replace that and the electrolytes lost by the diarrhoea (Blaser 1990). People with diarrhoea who are unable to take oral fluids due to nausea may need medical attention and intravenous fluids, especially in young children. Since the disease is self-limiting,

antibiotic or antimicrobial therapy is usually not indicated, unless in severe cases of the disease where systemic involvement appears to be present (Dryden *et al.* 1996; Blaser *et al.* 1995; Ruiz *et al.* 1998). This however is only for the doctor or specialist to decide, and should not be taken without their consultation. It is believed that antidiarrhoeal medications are generally not given as they may prolong the infectious disease, and this could lead to serious complications (Blaser 1997).

2.3 Miscellaneous

South Africa and Africa on the whole are faced with high levels of poverty and malnutrition and as expected these victims would generally have compromised or weaker immune systems. As such, these people stand a better chance of contracting diseases than healthy individuals would. Another disadvantage is that there are no formal reporting systems in place in order to document any sporadic cases or outbreaks of diseases. Hence, only until such infrastructure is in place can investigations determine the prevalence of different diseases in this and other related countries. However, governments responsibility in this predicament should be to educate consumers about so called “safe” and “unsafe” foods and to educate people about proper handling, cooking and storage of food.

2.3.1 Target Populations

Anyone can be affected by *Campylobacteriosis*. The disease frequently affects children, young adults, the elderly and especially those with a suppressed immunity (Anderson *et al.* 2001). The incidence of *Campylobacteriosis* in HIV-infected patients is higher than in the general population. For example, in Los Angeles county between 1983 and 1987, the

reported incidence of *Campylobacter* in patients with Aids was 519 cases per 100, 000 people. That is 39 times higher than in the general population (Perlman et al., 1988). Travellers are more prone to be infected due to changes in climate and environment, a condition known as Traveller's diarrhoea. Generally though, any person with poor personal hygiene can contract the disease easily.

2.3.2 Selected Outbreaks

The biggest outbreak of *Campylobacteriosis* recorded due to *C. jejuni* occurred in Bennington in the United States, where approximately 2000 people were affected. It was later discovered that the source of infection was the non-chlorinated water used as the common water supply. Smaller outbreaks usually occur amongst children taking class trips to dairies and consuming raw milk (Hopkins *et al.* 1984). In April 1986, in the United States, an elementary school child was cultured for bacterial pathogens, due to bloody diarrhoea. The laboratories had isolated *C. jejuni*. Questionnaires were distributed amongst the children and staff at school. Of the 172 students, 32 reported diarrhoea, 16 reported nausea, 9 reported fever, 8 reported vomiting, and 4 reported bloody stools. Milk was implicated because the dairy had pasteurised the milk at 10°C lower than the specified temperature for 5 minutes less than the specified time. The surplus milk that was sent to the school had a high somatic cell count. The milk was traced back to the cows from the herd that had *C. jejuni* in their faeces (Schmid *et al.* 1987). This incidence therefore emphasises the need to adhere to strict control measures, especially pasteurisation times and temperature standards.

2.3.3 Prevention – Tips for preventing *Campylobacteriosis*

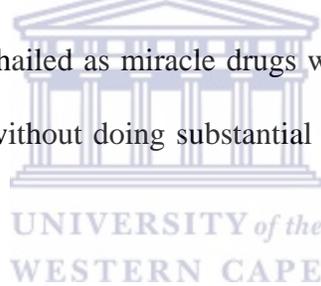
The two basic ingredients for preventing infection is good personal hygiene and some common sense. In order to prevent infection, the following simple food handling and safe food storage practices need to be obeyed: (Available: <http://hna.ffh.vic.gov.au/phb/hprot/idci/camp.html>)

- Cooked foods cannot be placed on surfaces or dishes that contained raw meat or poultry.
- Hands need to be washed after handling all raw meats. Washing and drying almost completely removes a huge load of cells from surfaces.
- Food handlers should dry their hands as wetness and surface fluids allows protection of *C. jejuni*, thereby causing auto-infection. It is essential to use disposable paper towels or an air dryer to dry hands as cloth towels get dirty quickly and can spread germs between persons.
- Hands should be washed before eating and after using the toilet or changing nappies.
- Pets are carriers of *Campylobacter* and any contact with them demands cleaning up.
- Raw and cooked foods can be handled with the same implements and utensils provided they have been washed between uses.
- Kitchen surfaces and equipment must be kept clean at all times.
- Foods can be defrosted by placing it on only the lower shelves of a refrigerator.
- Most importantly, all raw foods must be cooked thoroughly.
- Cooked foods must be refrigerated as soon as it gets cold.

- Raw foods are to be refrigerated below cooked foods to prevent cross-contamination.
- All raw vegetables must be thoroughly washed before eating.
- Food must be reheated until the internal temperature reaches at least 75 °C
- Consumption of unchlorinated/untreated surface water and raw or semi-pasteurised milk should be completely avoided.

2.4 Antibiotics and Antimicrobials

Antimicrobials and antibiotics first burst on the scene in the 1940's, and a new revolution in medicine began. They were hailed as miracle drugs when doctors discovered that they could kill bacteria in the body without doing substantial harm to the body itself (Alcama, 1994).

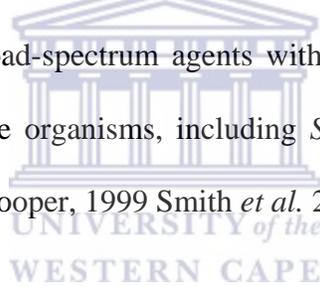


Antimicrobials are chemical substances used within the human body for therapeutic purposes. The term generally implies a chemical that has been synthesized by chemists or produced by a modification of a preexisting chemical. By contrast, an antibiotic is a product of the metabolism of a microorganism, although many are currently produced by synthetic or semisynthetic means. Antibiotics that are derived from a certain organism are safe to the organism, but inhibitory to other organisms (Alcama, 1994).

There are currently about 22 different classes of antimicrobials and antibiotics but only one class of antimicrobials, the quinolones and fluoroquinolones, have been emphasized on.

2.4.1 Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones are antimicrobial agents effective in the treatment of selected community-acquired and nosocomial infections. They are bactericidal and exhibit concentration-dependant killing. Quinolones target bacterial DNA gyrase and topoisomerase IV, the enzymes essential for DNA replication and transcription (Smith *et al.* 2001). Quinolones however had displayed poor systemic distribution and limited activity against gram negative bacteria whereas its next generation, the fluoroquinolones, proved to be more readily absorbed and had an increased activity against gram-negative bacteria. Newer fluoroquinolones are broad-spectrum agents with enhanced activity against many gram-negative and gram-positive organisms, including *Streptococcal* and *Staphylococcal* species (Goldstein *et al.* 1999; Hooper, 1999 Smith *et al.* 2001).



2.4.1.1 Description

A quinolone called nalidixic acid is a broad spectrum antimicrobial that blocks DNA synthesis in certain Gram-negative bacteria that cause urinary tract infections. Synthetic analogues of nalidixic acid called fluoroquinolones are the most recently approved class of antibiotics and nothing comparable is expected to become available for several years. Since their introduction for clinical use in the late 1980's; they have been used successfully for a large number of clinical situations. They were excellent agents for treatment of complicated urinary tract infections as well as for gonorrhoea and chlamydia and for intestinal infections due to gram-negative bacteria such as *Salmonella*, *Shigella*, and

Campylobacter (Wistrom and Norrby, 1995; Skirrow *et al.* 1995). Fluoroquinolones have also been used in the prevention and treatment of a variety of ocular infections. These infections include conjunctivitis, corneal ulcers, and endophthalmitis (Leeming, 1999). Examples of fluoroquinolones are ciprofloxacin, enrofloxacin, ofloxacin, norfloxacin and lomefloxacin. Newer fluoroquinolones on the market include levofloxacin, sparfloxacin and trovafloxacin (Blondeau, 2004; Anderson *et al.* 2001).

2.4.1.2 Administration and Distribution

Quinolones and fluoroquinolones are administered orally but some can be given intravenously for treatment of serious infections. In the United States, nine fluoroquinolones are currently approved for human use. These are Norfloxacin (1986), followed by ciprofloxacin (1987), ofloxacin (1990), enoxacin (1991), lomefloxacin (1992), levofloxacin (1996), trovafloxacin (1997), gatifloxacin (1999), and movifloxacin (1999) (Anderson *et al.* 2001).

Apart from its extensive use in treatment of human illnesses, fluoroquinolones too have been used in treating diseased farm animals and poultry. Furthermore, subtherapeutic levels of fluoroquinolones are incorporated into animal feeds to improve feed efficiency and stimulate growth (van Diest and de Jong, 1999). Fluoroquinolone use in poultry in the United States began in 1995, and since then several other countries have introduced antimicrobials in animal feeds as observed in the table below: (van Diest and de Jong, 1999).

Table 2.2: Quinolones licensed for use in food animals by region of the world (van Diest and de Jong, 1999)

Region	Livestock	Poultry	Pet animals	Fish
Europe	Enrofloxacin, flumequine, marbofloxacin, danofloxacin	Enrofloxacin, difloxacin, flumequine, oxolinic acid	Enrofloxacin, difloxacin, marbofloxacin	Sarafloxacin, (oxolinic acid) ^a
USA	None	Enrofloxacin, sarafloxacin	Enrofloxacin, difloxacin, orbifloxacin	None
Japan	Enrofloxacin, danofloxacin, orbifloxacin, difloxacin, oxolinic acid	Enrofloxacin, danofloxacin, ofloxacin, vebufloxacin, oxolinic acid	Enrofloxacin, orbifloxacin	Oxolinic acid
Asia	Enrofloxacin, danofloxacin, ciprofloxacin	Enrofloxacin, ciprofloxacin danofloxacin, flumequine, norfloxacin, oxolinic acid, (sarafloxacin)	Enrofloxacin	Oxolinic acid, Enrofloxacin, flumequine
Canada		enrofloxacin	Enrofloxacin	

Region	Livestock	Poultry	Pet animals	Fish
Australia	None	none	Enrofloxacin	None
South Africa	Enrofloxacin, danofloxacin	Enrofloxacin, danofloxacin, norfloxacin	Enrofloxacin	
^a Substances in parentheses are in limited use; ^b Voluntarily withdrawn from the market in 1998.				

2.4.1.3 Use in Agriculture

The data presented in table 2.2 indicate that quinolones are used for treatment of animal disease in many parts of the world and in some regions they are also used for disease prevention (van Diest and de Jong, 1999). Fluoroquinolones are used as growth promoters so as to enhance the growth rate of the animals. Subtherapeutic levels of fluoroquinolones are incorporated into animal feeds to improve feed efficiency and stimulate growth (Sam *et al.* 1999). As growth promoters, antimicrobials allow farm animals to gain 4 to 5 % more body weight. Hence the cost of production would increase substantially. However, it has been observed under experimental conditions that quinolone treatment of *Campylobacter* – colonized broiler chickens has induced quinolone resistance in the bacteria (Jacobs Reitsma *et al.* 1994). Hence, antimicrobial usage in agriculture has led to the emergence of resistance in bacteria present in the gut of animals consumed as food (Engberg *et al.* 2001).

2.4.1.4 Market Dominance and Structural Evolution of Fluoroquinolones

The newer fluoroquinolones as a class possess many characteristics that make them useful antimicrobial agents against gram-negative and gram-positive organisms. These include, their broad spectrum of activity, good oral absorption and tissue penetration, relatively long serum elimination half-lives that allow once or twice daily dosing, predictable drug-drug interactions, and a relatively low incidence of serious side effects.

Fluoroquinolones are reported to be extremely effective synthetic antimicrobials that were approved in the United States for treatment of respiratory and enteric infections of poultry in 1995 and cattle in 1998 (Anderson *et al.* 2001). A wide variety of serious bacterial infections in humans are treated with fluoroquinolones, including serious systemic infections in immunocompromised patients and those that resist treatment with more traditional antibiotics as a result of a broad spectrum of organisms causing the infection (Anderson *et al.* 2001). Fluoroquinolones also represent a class of antibacterials which are effective against a large population of gram negative and anaerobic species responsible for ocular infections (Leeming, 1999).

The broad spectrum of activity of fluoroquinolones is owed to their development over the years from a first-generation class of antimicrobials to a fourth-generation class. The first quinolone, specifically nalidixic acid, was just a basic synthetic molecule to treat uncomplicated urinary tract infections (Norris and Mandell, 1998; Smith *et al.* 2001). To expand the spectrum of activity, structural modification of the first-generation quinolone

was called for by the addition of side-chain chemical structures along the quinolone skeleton.

The development of second-generation quinolones such as oxolinic acid and cinoxacin brought about substantial improvements in gram-negative microbial coverage, including *antipseudomonal* activity. Yet clinical use of these agents remained limited due to the risk of renal toxicity (Appelbaum and Hunter, 2000; King *et al.* 2000).

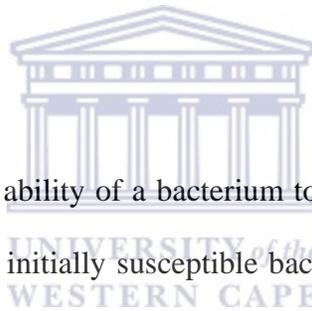
Third-generation quinolones were born with the fluorination of the quinolone structure giving rise to the classification of the future compound, the fluoroquinolones. The property of fluorination accommodated the activity of these antimicrobials against gram-positive organisms and ultimately their clinical uses in the field of ophthalmology (Ball *et al.* 1998). The third-generation quinolones, now called fluoroquinolones, included the groups of norfloxacin, ofloxacin, ciprofloxacin and levofloxacin. Norfloxacin displays good activity against gram-negative bacilli but limited activity against gram-positive bacteria (Blondeau, 2000). Ciprofloxacin, on the other hand, was modified further to improve the efficiency of antimicrobial activity against gram-positive organisms especially in ocular infections (Fisher and Heaton, 2003). Ofloxacin exhibited activity against anaerobes such as *Propionibacterium acnes* (Mather *et al.* 2002). Levofloxacin conferred efficient activity against strains such as *Streptococcus pneumoniae* and *Streptococcus viridans* (Appelbaum and Hunter, 2000; Mather *et al.* 2002).

Finally the development of fourth-generation compounds such as moxifloxacin and gatifloxacin became necessary due to the growing resistance towards the third-generation compounds by especially gram-positive organisms (Mather *et al.* 2002). The modifications

made to the quinolone skeleton to develop moxifloxacin and gatifloxacin are believed to allow for the dual mechanism of action in gram-positive organisms, in addition to reducing its efflux from the bacterial cell, thereby improving the spectrum of activity of these agents especially against strains of *Streptococcus* and *Staphylococcus* (Fukuda *et al.* 2001; Mather *et al.* 2002; Ruiz *et al.* 2005).

Hence, the versatility of fluoroquinolones have certainly stood out amongst other classes of antimicrobials due to their facilitating changes and modifications to enhance improvement in activity against both gram-positive and gram-negative organisms.

2.5 Antibiotic Resistance



Antibiotic resistance defines the ability of a bacterium to resist or overcome the effects of an antibiotic. This occurs when initially susceptible bacteria or other microbes that cause disease evolve into forms that can no longer be killed by antibiotics. The susceptible cells die, the more resistant cells survive and proliferate giving rise to a new population of resistant organisms. The process happens faster when antibiotics are used very frequently, especially at low doses over long periods of time, which is common on livestock and poultry farms where antibiotics are added to feed (Anderson *et al.* 2001).

2.5.1 Concept of Resistance

Of all antibiotics or antimicrobials available today, not one is completely effective against all bacterial pathogens. Bacteria with their sole purpose of proliferating and surviving

possess certain characteristics or defense mechanisms to facilitate their survival. Upon exposure to antibiotics, they develop resistance to them either by:

(I). **Intrinsic Resistance:** Some bacteria are intrinsically resistant to certain of the antibiotics by possessing antibiotic resistant genes either in their chromosome or as part of plasmids carried by them (Alcama, 1994).

(II). **Acquired Resistance:** Many bacteria, on the other hand, acquire resistance to one or more of the antibiotics to which they were formerly susceptible. They develop resistance by acquiring genes encoding proteins that protect them from the harmful effects of the antibiotic. In some cases the genes arise by mutation; in others, they are acquired from other bacteria that are already resistant to the antibiotic. The genes are often found on plasmids which spread easily from one bacterium to another (Alcama, 1994).

An alarming number of human pathogens (including strains of *Streptococcus*, *Salmonella* and *Campylobacters* have acquired genes to combat most of the presently used antibiotics. These multi-drug resistant strains are particularly common in hospitals where antibiotic use is heavy, and the patients often have weakened immune systems (Alcama, 1994). Multi-drug resistance often occurs when organisms are exposed to more than one kind of antibiotic either at the same time or at different times. Studies conducted in Japan, Denmark and in England have already reported the findings of multi-resistant *C. jejuni* and *C. coli* isolates to important antimicrobials such as tetracycline, erythromycin and chloramphenicol (Andersen *et al.* 2005; Ishihara *et al.* 2004; Kramer *et al.* 2000).

Apart from acquiring genes to combat antibiotics, there are other ways bacteria can overcome the targets of antibiotics, such as synthesising pumps in their plasma membrane

through which they remove antibiotics from the interior of the cell; or modifying the antibiotic so it can no longer recognise its target; or even modify the structure of their peptidoglycan wall and thus avoid the inhibitory effects of antibiotics. As in the case of defeating quinolones, some bacteria modify their DNA gyrase (Blondeau, 2004).

2.5.2 Fluoroquinolone Resistance

Resistance to quinolones and fluoroquinolones occurs through chromosomal mutations in the genes encoding DNA gyrase and Topoisomerase IV (Engberg *et al.* 2001). These enzymes are required for bacterial DNA synthesis. Other factors that may also be significant are porin and efflux mutations (Charvalos *et al.* 1995). The enzyme mutations result in an alteration of the target region where the drug binds to the enzyme, the drug exhibits reduced affinity for the target site and becomes ineffective (Kampranis and Maxwell, 1998). Mutations that result in alterations of the outer membrane porin proteins of gram-negative organisms lead to decreased permeability of the drug through the outer membrane so less drug reaches the target enzyme. Mutations that enhance the organism's efflux capability increase the amount of drug pumped out of the cell. In addition to this, other mutations may also dictate different levels of fluoroquinolone resistance (Blondeau, 2004).

2.5.3 Mechanism of Quinolone Resistance in *Campylobacter jejuni*

Quinolones and fluoroquinolones block the action of two bacterial enzymes, viz. DNA gyrase and Topoisomerase IV which are responsible for relieving the coils that form in DNA when the helix is being opened in preparation for replication, transcription or repair.

Eukaryotes have no DNA gyrase and hence fluoroquinolones are both specific and bactericidal (Hooper, 1999; Smith *et al.* 2001).

Topoisomerases separates the duplex bacterial DNA, inserts another strand through the break and then reseals the originally separated strands (Wang, 1996). DNA gyrase introduces negative superhelical twists in the bacterial DNA double helix ahead of the replication fork thereby catalyzing the separation of daughter chromosomes. This process is essential for initiation of DNA replication and allows for binding of initiation proteins. DNA gyrase consist of four monomeric subunits. These are two Gyr A and two Gyr B monomers encoded by the *gyr A* and *gyr B* genes respectively (Blondeau, 1999). Topoisomerase IV is responsible for decatenation thereby removing the interlinking of daughter chromosomes and allowing segregation into two daughter cells at the end of a round of replication (Zechiedrich and Cozzarelli, 1995). Topoisomerase IV consist of four homologous monomeric subunits, namely two Par C and two Par E subunits encoded by the *par C* and *par E* genes respectively (Kato *et al.*, 1993).

Fluoroquinolones binds to the enzyme-bound DNA complex (ie. DNA gyrase + bacterial DNA or Topoisomerase IV + bacterial DNA) thereby creating a conformational change that results in the inhibition of normal enzyme activity. The drug–enzyme–DNA complex blocks progression of the replication fork which inhibits normal cell DNA synthesis and what follows is rapid bacterial cell death (Kampranis and Maxwell, 1998; Marlans and Hiasa, 1997).

Resistance in *Campylobacter* arises when mutations occur in *gyr A* and only occasionally in the topoisomerase IV (*par C*) genes. Cloning and sequencing of the *C. jejuni* gyrase A

gene often demonstrated that mutations in *gyr A* at positions Thr-86, Asp-90, and Ala-70 were responsible for resistance (Wang *et al.* 1993; Ruiz *et al.* 1998). Mutations at Thr-86 are associated with a higher level resistance to nalidixic acid (MIC 64-128 ug/ml) and ciprofloxacin (MIC 16-64 ug/ml) than mutations at Asp-90 or Ala-70. *C. jejuni* isolates resistant to even higher levels of quinolones (ciprofloxacin MIC of 125 ug/ml) carry two mutations, one in *gyr A* Thr-86 and the other in the topoisomerase IV subunit par C at Arg-139 (Gibreel *et al.* 1998). Normally an organism is highly resistant to all quinolones after multiple mutations are induced in different genes or different parts of a gene. Generally though, it was observed that the DNA gyrase A gene purified from quinolone-resistant mutants of *C. jejuni* was 100-fold less sensitive to inhibition by quinolones than the wildtype gyrase A gene (Gootz *et al.* 1991). Mutations in the gyrase A subunit tend to cluster in a region known as the quinolone resistance-determining region (QRDR). The QRDR represents that portion of the *gyr A* gene where the most significant mutations ever recorded tends to occur, such as the Thr-86-Ile mutation most responsible for fluoroquinolone resistance (Gibreel *et al.* 1998)

In 1995, evidence was obtained that efflux of fluoroquinolones in *C. jejuni* does also exist (Charvalos *et al.* 1995). Multidrug resistant (MDR) efflux pumps in the membranes of the bacterial cells have been shown to reduce fluoroquinolone activity and contribute to low-level resistance by actively pumping out the drug from the bacterial cell (Hooper, 1999).

2.5.4 Link Between Veterinary Use of Fluoroquinolones and Resistant

Campylobacter Infections in Humans

In the 1980's, the introduction of fluoroquinolones offered a new approach to antibiotic intervention (Wistrom and Norrby, 1995). They were extremely effective antimicrobials that were approved in the United States for the treatment of respiratory and enteric infections of poultry in 1995 and eventually for cattle in 1998 (Anderson *et al.* 2001). Fluoroquinolones initially had good in vitro activity for thermophilic *Campylobacter* species, as well as for members of the family of Enterobacteriaceae in patients of both community-acquired acute diarrhoea and travelers diarrhoea (Piddock, 1995). However it was reported as early as 1991 that quinolone resistant *C. jejuni* and *C. coli* emerged from humans in the Netherlands. This coincided with the introduction of fluoroquinolones in veterinary medicine (Endtz *et al.* 1991). Fluoroquinolones are widely used in chickens and swine prophylactically and as a means to promote reproduction and weight gain (Velazquez *et al.* 1995).

Several studies have shown that food animals can be a substantial source of infection in humans and that the same serotypes and genotypes can be isolated from humans and food animals (Nielsen and Nielsen, 1999; On *et al.* 1998; Nielsen *et al.* 1997; Orr *et al.* 1995; Owen *et al.* 1999). Quinolones were approved for veterinary use in the United Kingdom and the United States in late 1993 and 1995, respectively, reports from these areas have since showed increasing quinolone resistance profiles (Smith *et al.* 1999; Piddock, 1998; Sam *et al.* 1999).

Whilst many researchers believe that veterinary use of fluoroquinolones gives rise to resistant cells in the gut of food animals, and ultimately in humans via the food chain, some believe that this is a negligible factor and thus most resistant cells are harbored within humans themselves through human use of the antibiotics. Theoretically it has been proven that horizontal transfers of resistant cells are absolutely possible and that cells whether resistant or sensitive are readily passed on from food animals to humans when humans consume the contaminated food. In fact, while human use of fluoroquinolones does contribute to an increase in the number of resistant cells; their relative contribution to an increase in resistance compared to the use of these agents in husbandry appears to be small (van Diest and de Jong, 1999). The table below represents the prevalence of fluoroquinolone resistance after the approval of fluoroquinolone use in livestock (Anderson *et al.* 2001).



Table 2.3: The Prevalence of Fluoroquinolone Resistance of Campylobacter in Human Isolates in Reporting Countries^a (Anderson *et al.* 2001)

Timepost-approval	Country	Resistance (%)	References	Model input (%)
Prior to introduction	US	1.3	Smith <i>et al.</i> 1999	1.3
1 year of FQ use	US	1	Smith <i>et al.</i> 1999	1-8
	Canada	3.5	Gaudreau and Gilbert, 1998	
	UK	4.1	Gaunt and Piddock, 1996	

Timepost-approval	Country	Resistance (%)	References	Model input (%)
	Netherlands	8	Endtz <i>et al.</i> 1991	
2 years	US	3	Smith <i>et al.</i> 1999	3-11
	Netherlands	6	Endtz <i>et al.</i> 1991	
	US	7.5	NARMS 1997	
	Netherlands	11	Endtz <i>et al.</i> 1990	
3 years	US	8	NARMS 1998	8-12.7
	UK	12	Threlfall <i>et al.</i> 1999	
	Canada	12.7	Gaudreau <i>et al.</i> 1998	
4 years	UK	12	Threlfall <i>et al.</i> 1999	12
7 years	Netherlands	11	Talsma <i>et al.</i> 1997	11
10 years	Netherlands	29	Talsma <i>et al.</i> 1997	29
Worst case scenario	Spain	>50	Gomez-Garces <i>et al.</i> 1995	50-84
	Thailand	84	Hoge <i>et al.</i> 1998	

^a The starting point is the approval of fluoroquinolones for livestock use. The resistance figures for the different countries are grouped together, based on the number of years since approval.

It is therefore imperative that licensing of antibiotics in veterinary use needs to be reconsidered and better control mechanisms needs to be in place in order to eliminate this route of transmission of resistant cells.

2.5.5 Cost of Antimicrobial Resistance

Treating resistance infections requires the use of more expensive or more toxic alternative drugs and longer hospital stays. Researchers found that patients infected with resistant *C. jejuni* cells had a longer duration of diarrhoea and illness than patients infected with sensitive isolates (Engberg *et al.* 2004; Nelson *et al.* 2004). In addition, it frequently means a higher risk of death for the patient harboring a resistant pathogen (Smith, 2000). Even with the development of new drugs, resistance to drugs is an increasingly important problem with certain pathogens (Smith *et al.* 1999). Deaths due to infectious diseases have been increasing in the United States in recent years. According to a 1996 report in the Journal of the American Medical Association, between 1980 and 1992, the death rate due to infectious diseases as the underlying cause of death increased by 58% in the United States. Some of this was due to an increase in the Aids epidemic during the period. Among those aged 65 and over, deaths due to infectious diseases increased 25% during the period 1980-1992. Thus there seems to have been a significant increase in deaths due to infectious diseases in the United States over the past 20 years (Shallow, 2000).

In Europe and the United States, diseases and deaths caused by pathogens are often reported due to the availability of important information hubs such as The Centers for Disease Control and Prevention (CDC), World Health Organisation (WHO), the National Antimicrobial Resistance Monitoring System (NARMS) and the Food and Drug Association (FDA). Here disease epidemics and even sporadic cases of a disease are frequently reported as all important reporting systems and strategies are in place in order to do so.

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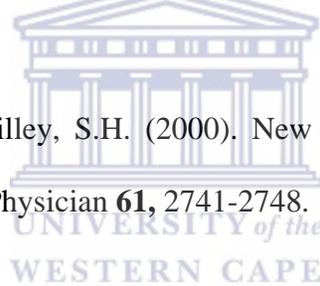
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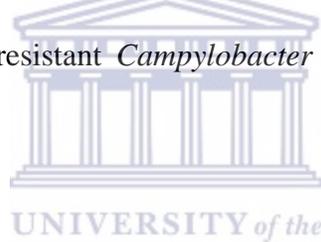
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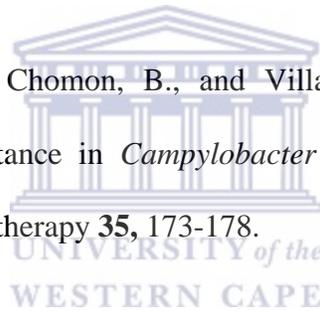
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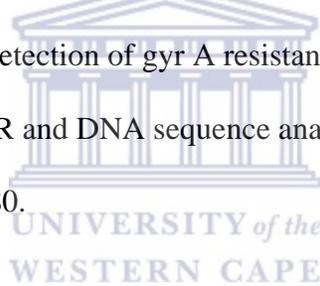
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CHAPTER 3

Differentiation between Quinolone Resistant and Sensitive isolates of *Campylobacter jejuni* by a Multiplex PCR assay

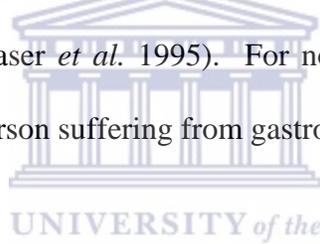
3.1 Abstract

In *Campylobacter jejuni* the target gene of the quinolones or fluoroquinolones is the DNA gyrase which, when inhibited, disrupts DNA replication and repair; transcription and other cell processes involving DNA (Alcamo, 1994). Several mechanisms may be involved in the conversion from quinolone susceptibility to quinolone resistance but the most important seems to be the acquisition of the thr-86-ile mutation in the quinolone resistance determining region (QRDR) of the gyrase A gene. It was reported that this mutation was the only one that significantly appeared on comparison between the two antibiotic biotypes in *C. jejuni* (Gibreel *et al.* 1998). Forty *C. jejuni* clinical isolates of which 25 were quinolone resistant and 15 sensitive were obtained from the Red Cross Children's Hospital in Cape Town. Three primers were designed to detect the difference between the resistant and sensitive types by targeting the mutation at codon 86 in the gyr A gene. In this study, the PCR procedure produced two bands for the resistant isolates, but only a single band for the sensitive ones. One primer in the multiplex PCR assay, the mutation detection primer, was responsible for recognising the point mutation mentioned above that are present in only resistant *C. jejuni* isolates. In so doing, the resistant and sensitive types were distinguished not only in clinical isolates, but also in poultry and abattoir samples.

3.2 Introduction

Campylobacter jejuni (*C. jejuni*) is currently the leading cause of gastroenteritis in humans worldwide (Zirnstein *et al.* 1999). *C. jejuni* are harboured in the gut of food animals through which humans become contaminated either by ingestion of contaminated meat or by exposure to surfaces or environments on which *C. jejuni* resides (Coates *et al.* 1987; Norcross *et al.* 1992).

The need for differentiation between resistant and sensitive profiles of *C. jejuni* stems from the fact that resistance to antibiotics enhances the pathogenicity of *C. jejuni* cells upon infection. These resistant pathogens cause a longer duration of symptoms as well as more severe forms of the disease (Blaser *et al.* 1995). For no treatment available, this would mean great discomfort for the person suffering from gastroenteritis.



The objectives are therefore to firstly identify the pathogen, and then the resistance profile of the pathogen concerned before decisions are to be made in terms of possible treatment and medication for the diseased patient.

In public health facilities, there is a demand for rapid results, especially with the screening of a large number of cases on a daily basis. This demand calls for a technique that could promise rapid and very reliable results in a short a time as possible. Polymerase Chain Reaction (PCR) has been acknowledged as the one technique that is efficient and fast in cases where time is of the essence.

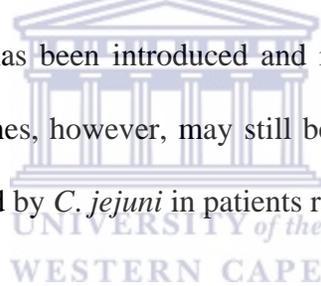
The Red Cross Children's Hospital in Cape Town, South Africa is the only hospital in sub-Saharan Africa that focuses exclusively on diseases prevalent in children. The hospital is a reputable information hub where cases of gastroenteritis in children are reported timeously and correctly. Cases of patients attending day clinics and public health facilities often go unreported and as such, much information about the incidence of cases of gastroenteritis in South Africa is lost.

Technical staff at the hospital focused on the isolation and identification of the pathogens after which their resistant profiles were established. Resistance screening was done by the antibiotic disc diffusion test and once established, all quinolone resistant and sensitive *C. jejuni* were sent to the Food Microbiology laboratory of the University of the Western Cape for further testing. For the purpose of this study, researchers at the university attempted to design a standard PCR procedure to directly differentiate between resistant and sensitive isolates of *C. jejuni* from clinical samples grown on tryptose blood agar (TBA) (Oxoid, CM233; Biolab C48) media.

The aim, therefore, was to use this PCR profile of clinical samples as a standard against which isolates from poultry and abattoir samples could be compared. The presence of pathogens on foods is often a nightmare for the food industry as it is their responsibility to ensure the complete removal of dangerous contaminants of their products. Consumers on a wide scale, unfortunately, are not very much aware of the potential risks involved in consuming "unsafe" foods. It is therefore up to the producer and retailer to ensure that food is completely safe before its distribution on shelves. The poultry industry worldwide has proven to be the most important factor responsible for many food-borne illnesses (Bryan and Doyle, 1995). Several critical control points are needed to be taken care of to ensure

all hazards are removed and is often one or more of these factors that are overlooked that results in an unsafe food. Now, a new serious underlying problem is at hand and that is the introduction of antimicrobials into animal feeds for the purpose of speeding up growth of animals and chickens (Sam *et al.* 1999). The problem of antibiotic resistance settles in and animal carcasses now not only carries harmful pathogens, but pathogens that are harder to destroy and to ultimately remove. The result is transmission of these resistant bugs to humans and ultimately disease fatalities and mortalities (Engberg *et al.* 2001).

This study is therefore two-fold in that firstly it can be ascertained whether any *Campylobacter jejuni* pathogens are present, and secondly if these pathogens present are sensitive to quinolones or not. Once resistant isolates are confirmed, one can investigate where the factor of resistance has been introduced and immediately try to eliminate this route of transmission. Quinolones, however, may still be saved to remain the last line of defense for gastroenteritis caused by *C. jejuni* in patients residing in South Africa.



3.3 Materials and Methods

3.3.1 Isolation of *Campylobacter jejuni*:

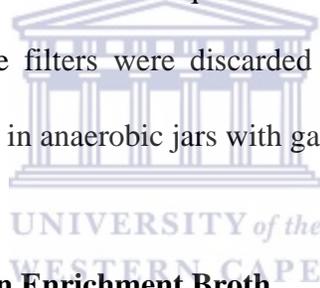
3.3.1.1 From stools:

Fresh tryptone blood agar (TBA) (Oxoid CM 233, Basingstoke UK) plates were prepared and enriched with 10% lysed horse blood obtained from the Delft Farm (MRC) in Bellville, Cape Town. The stool samples were diluted by serial dilution in 10% saline water. 200ul of the stool emulsion was used to flood the centre of 0.4um pore size membrane filters (ME

26, Schleicher and Schuell) placed onto the TBA plates. The filter paper was removed and discarded after 15 minutes. The plates were then inverted and incubated in anaerobic jars with gas packs (Oxoid BR 38) for 2 to 3 days at 37°C or 42°C.

3.3.1.2 From Poultry Caeca:

The procedure for the isolation of *C. jejuni* were repeated for 7; 14; 21 and 28 day old chickens. TBA plates were prepared with 10% horse blood. The skin of the caeca was cut or slit to release the contents. The contents was swabbed and gently spread across a few blood plates. In addition, the caeca contents was swabbed and placed in 5ml of 10% saline water. This was well shaken and 200ul was squirted onto the centre of 0.4um membrane filters on the TBA plates. The filters were discarded after 15 minutes and the plates incubated for 2 to 3 days at 37°C in anaerobic jars with gas packs.



3.3.2 Pre-enrichment in Bolton Enrichment Broth

Swabs were used for collecting the contents of the poultry caeca. The swabs were placed into 5ml Bolton Enrichment Broth (Oxoid SR 183). The swabs were then cut to within the height of the containers, the containers sealed and incubated for 4 hours at 37°C and then for 24 hours and 48 hours respectively at 42°C in anaerobic jars with gas packs. Finally, 200ul of emulsion were subcultured onto marked TBA plates for 48 hours at 37°C or 42°C.

3.3.3 Biochemical Tests

Microscopic examinations were done on pure cultures for identifying the shape, size and darting motility. Further biochemical testing involved hippurate hydrolysis, catalase and indoxyl acetate hydrolysis tests to confirm *Campylobacter jejuni*. Best result for all tests were obtained by using 48 hour old cultures. All enzymes are normally better developed in H₂.

3.3.3.1 Hippurate hydrolysis

The hippurate hydrolysis test allows for only *C. jejuni* species to be positive. A stock consisting of 5% hippuric acid (sodium salt; Sigma H 9380; Merck 820648) was obtained of which 25 ml of this acid was added to 100 ml of distilled water to prepare hippurate broth. This broth was then filtered and placed into glass tubes of about 1 ml amounts and stored in the freezer, covered, until further use. Autoclaving was not required.

Separately, a ninhydrin solution was prepared by adding 3.5g of Ninhydrin (Merck 6762, BDH 10132 4E) to 100 ml of a 50:50 ratio of Butanol in Acetone solution. This was also stored in a freezer until further use.

The test was performed by firstly thawing the tubes of hippurate broth and then inoculating them heavily with the organism of interest. The tubes were incubated overnight (which was preferential to a 2 hour test requiring atleast a 30 minute incubation period). Upon completion of incubation, a 0.5 ml of the thawed ninhydrin solution was gently added to the tubes. It was important not to shake or aerate the tubes. The results were recorded within

10 minutes and was interpreted as purple for positive and colourless or light purple for negative.

The colour change is due to the fact that hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidizing agent, ninhydrin. Ninhydrin becomes reduced in the process and the end products of the ninhydrin oxidation form a purple-coloured dye.

3.3.3.2 Catalase Test

A loopful of culture, taken carefully from a tryptose blood agar plate, was picked up with a capillary tube containing hydrogen peroxide (20 volumes). Oxygen bubbles were trapped in the capillary tube. This was indicative of the presence of *C. jejuni* and hence a positive test since catalase released by the cells break down the hydrogen peroxide into oxygen and water.

A positive reaction can be so strong that large bubbles are formed that can very successfully block the capillary tube and prevent the ascent of any bubbles. On close scrutiny the trapped bubbles can be seen on the surface of the culture or forced out at the bottom.

3.3.3.3 Indoxyl Acetate Hydrolysis

For this test a 10% (wt/vol) solution of indoxyl acetate (Sigma, 13500) in acetone is prepared. Filter paper strips are saturated with this solution and then air-dried. These were stored in amber bottles in the fridge until further use. A loopful of culture were then rubbed

onto a small area of the strip. This is followed by the wetting of the strip with distilled water, keeping the strip moist for up to 10 minutes. The development of a dark blue colour is indicative of a positive test for *C. jejuni*. The colour reaction is based on the fact that *C. jejuni* produces an esterase that, in the presence of oxygen, will hydrolyse the indoxyl present on the strip.

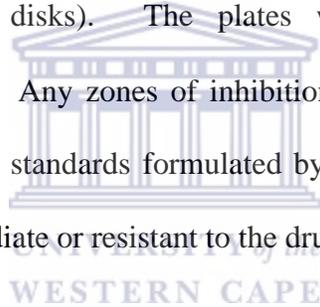
3.3.4 Molecular Tests

Molecular testing entailed the design of five primers for the Polymerase Chain Reaction procedure or PCR, two of which were designed specifically for differentiating between *Campylobacter jejuni* and the other species of *Campylobacter* in the genus, and three of which were used in a multiplex PCR procedure to differentiate between quinolone/fluoroquinolone resistant and sensitive isolates of *Campylobacter jejuni* isolates only. The polymerase chain reaction is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. The segments of DNA or parts of a gene against which the primers were designed are available in the database in genbank, available through the South African National Bioinformatics Institute at the University of the Western Cape.

3.3.5 Antimicrobial Resistance Testing

Antimicrobial resistance testing was done by the use of antibiotic diffusion disks in the agar disk diffusion method. This method has been standardized by the National Committee for Clinical Laboratory Standards (NCCLS). Tryptose Blood Agar plates were streaked with a pure culture of *Campylobacter jejuni* to obtain isolated colonies. The plates were incubated

microaerophilically at 37°C for 2 days or until well isolated colonies were to be obtained. Microaerophilic incubation was facilitated by the use of anaerobic Gas Packs (Oxoid BR 38) fitted into the glass jars containing the plates. The Gas Pack sachets were filled with 10 ml of distilled water upon opening and the jar was sealed rather tightly. The jars were incubated in a 37°C incubator for the entire 2 to 3 days. Four to five isolated colonies were eventually transferred with a loop to a tube of sterile saline and vortexed thoroughly. The bacterial suspension was then compared against a 0.5 McFarland standard and adjusted accordingly. Mueller-Hinton plates were inoculated evenly with this bacterial suspension using a sterile cotton wool swab. The disks containing known concentrations of one or more antibiotic types were directly placed onto the agar plates. (Plates were not to be overcrowded with too many disks). The plates were then incubated at 37°C, microaerophilically, for 2 days. Any zones of inhibitions were measured and compared with the zone-size interpretative standards formulated by the NCCLS. The isolates were recorded as susceptible, intermediate or resistant to the drug tested.



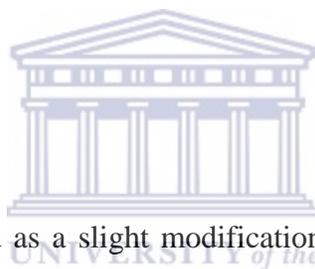
For *Campylobacter* the following standards have been suggested by the NCCLS for the quinolones.

Table 3.1: Zone size interpretative standards for *Campylobacter* for Quinolones / Fluoroquinolones

Antimicrobial agent	Disk potency (ug)	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ciprofloxacin	5	<15	16-20	>21
Nalidixic acid	30	<13	14-18	>19

Source: National Committee on Clinical Laboratory Standards (NCCLS), 1998

3.3.6 DNA Extraction



The following method was used as a slight modification to the DNA extraction protocol obtained from Molecular Cloning (Vol. 1; 3rd edition) titled “Preparation of Plasmid DNA.” Pure colonies were scraped off plates and resuspended in 0.5ml TE buffer (50mM Tris, 50mM EDTA, pH 8). This suspension was centrifuged at 14000 g for 10 minutes. The supernatant was poured off and 500ul of TE was added again to resuspend cells. Cells were lysed by adding 50ul of lysozyme (used 10mg of lysozyme in 1ml of sterile water). Gentle mixing was required for cell lysis to take place. The tubes were incubated at 37°C for 30 minutes. 50ul of a solution containing 10mg Proteinase K per ml in TE buffer and 20ul of 10% SDS were added to the above solution. The solutions were mixed and incubated further at 56°C for 2 hours or overnight at 37°C. Precipitation of proteins and cell debris was done with the use of 297ul of 3M Potassium acetate (pH 4.8). The tubes

were vortexed vigorously and then centrifuged at 16000 g for 10 minutes. The supernatant were added to 0.54 volumes of isopropanol and mixed gently. Again centrifuging at 16000 g for 10 minutes was required. The supernatant was poured off and the pellets were washed twice in 600ul of 70% ice cold ethanol. A final centrifugation at 13000 g for 2 minutes was required and the supernatant was poured off. The pellets were left to dry in the oven and when dry was resuspended in 50ul of DNA Rehydration solution (Wizard Genomic Purification Kit; Promega, 10mM Tris, 1mM EDTA). Tubes were stored at 4°C for short-term and -20°C for long term storage.

3.3.7 Primer design and Polymerase Chain reaction (PCR)

3.3.7.1 The following two primers were designed for the purpose of differentiating *Campylobacter jejuni* from all other species of *Campylobacter* in the genus. The primers were designed against the gyrase A gene of *Campylobacter jejuni* NCTC 11168 sequence available in the database in genbank (resources available at the South African National Bioinformatics Institute/ SANBI at the University of the Western Cape). (NB. Primer design programmes include Blast {Entrez, NCBI} and BCM Search Launcher accessed via www.sanbi.ac.za/links.html). A product of 480bp were amplified by the forward and reverse primers listed in the table below:

Table 3.2: List of primers used for confirmation of *Campylobacter jejuni* isolates

Primer	Sequence 5' to 3'	Direction
Naz 1	TTA GCT GAT GCA AAA GGT TA	Forward
Naz 2	GA GTA CTC TTT CAA ATG AGT	Reverse

The following reagents were used in the reaction mix for PCR assay 3.3.7.1:

Table 3.3: List of reagents and volumes in PCR tube

Reagents*	Volume (ul)
MgCl ₂ (25mM)	4.5
Sterile water	3.0
Primer 1 (5uM)	2.5
Primer 2 (5uM)	2.5
dNTP'S (2.5mM)	2.0
Taq polymerase (5U/ul)	0.1
DNA	1.0
10 x reaction buffer	2.5
Sterile water	6.9
Total	25

All PCR reactions were performed in a Thermal Cycler (Perkin Elmer/ Applied Biosystems, 2400).

The following conditions were used for a complete PCR cycle: The initial denaturation was at 94°C for 60 seconds, and was followed by 30 cycles of 94°C for 60 seconds (denaturation), 50°C for 30 seconds (annealing), and 72°C for 60 seconds (polymerization). The reaction was terminated with a final elongation at 74°C for 5 minutes. The PCR products were separated on 2% agarose gels in 0.5xTBE buffer and stained with ethidium bromide

3.3.7.2 The quinolone resistance determining regions of the gyrase A genes of *Campylobacter jejuni* isolates were displayed in the database in genbank (L04566 *C. jejuni* gyr A gene). From these regions, three specific sequence domains were chosen as primers for amplification of a 368 bp PCR product and a 265 bp product in a multiplex PCR assay. These primers have previously been used singly in individual PCR assays in a previous study by Gerald Zirnstein and colleagues published in the Journal of Clinical Microbiology in October 1999 (“Ciprofloxacin Resistance in *Campylobacter jejuni* Isolates: Detection of gyr A Resistance Mutations by Mismatch Amplification Mutation Assay PCR and DNA Sequence Analysis”). Hence, new conditions needed to be established for these primers to function optimally together in a single multiplex PCR assay. The table below lists the primers used in this study, as well as their respective nucleotide bases:

Table 3.4: List of Primers Used In This Study (Zirnstein *et al.* 1999)

Primer	Sequence 5' to 3'	Direction
Neyr A1 (primer A1)	CAG TAT AAC GCA TCG CAG CG	Reverse
Neyr A2 (primer A2)	TTT TTA GCA AAG ATT CTG AT	Forward
Neyr A3 (primer A3)	CAA AGC ATC ATA AAC TGC AA	Reverse/Mutation Detection primer

Primers A1 and A2 are conserved reverse and forward primers respectively and their function is to amplify a 368 bp PCR product within a wildtype *C. jejuni* isolate. Primer A3, on the other hand, is a reverse mutation detection primer which together with forward primer A2 amplifies a 265 bp product. This amplicon is indicative of a resistant isolate, which therefore carries the mutation frequently implicated in quinolone/ fluoroquinolone resistance. It is advisable, however, to confirm isolates as being *C. jejuni* (and not *C.coli*)

as the 3' end of the mutation detection primer, designed for the Thr-86-to-Ile mutation in codon 86 of the *C. jejuni* gyr A gene, is homologous to codon 86 of the wildtype *C. coli* gyr A gene and could generate a false positive product (Zirnstein *et al.* 1999). Re-confirmation of *C. jejuni* can be done by PCR using the primers listed in Table 3.4.

The following reagents were used in the reaction mix for PCR assay 3.3.7.2:

Table 3. 5: List of reagents and volumes in PCR tube

Reagents*	Volume (ul)
MgCl ₂ (25mM)	4.5
Sterile water	3.0
Primer A1 (5uM)	2.5
Primer A2 (5uM)	2.5
Primer A3 (5uM)	2.5
DNTP's (2.5mM)	4.0
Taq polymerase (5U/ul)	0.1
DNA	1.0
10 x reaction buffer	2.5
Sterile water	2.4
Total	25

*refer to appendix for reagent concentrations

The following conditions were used for a complete PCR cycle: The initial denaturation was at 94°C for 3 minutes, and was followed by 30 cycles of 94°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing), and 72°C for 20 seconds (polymerization). The reaction was terminated with a final elongation at 72°C for 5 minutes.

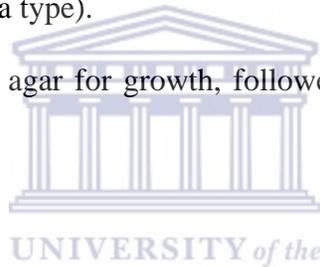
Upon completion of the PCR run, 8ul of each PCR sample plus 5ul of loading buffer was loaded onto a 2% agarose gel prepared in 0.5 x TBE. The molecular marker used in all experiments was 0.8ug of a 100 bp DNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; available from Promega). The gel was run by electrophoresis and results recorded for each isolate tested.

3.3.8 Negative Controls

The negative controls used in this study included:

Pseudomonas, Xanthomonas, Vibrio, Bacillus cereus, Shigella dysentery and Escherichia coli (Organisms of various genera type).

These were plated onto nutrient agar for growth, followed by DNA extractions and PCR with primers A1, A2, and A3.



Another set of only *Campylobacter* negative controls included:

C.fetus, C. lari, C. conciscus, C.sput.fecalis, C. mucosalis, C.coli, C.f. veneralis, C. fecalis, C. upsaliensis, C. curvus (Organisms of various specie types).

These were plated onto tryptose blood agar for growth, followed by DNA extractions and PCR with primers A1, A2, and A3.

3.3.9 Sample Size

Collectively 40 clinical isolates of *Campylobacter jejuni* were used for PCR testing which included 25 resistant isolates and 15 sensitive isolates. These were employed for setting up a standard for comparison with poultry and abattoir samples.

For poultry samples from a poultry farm, 16 isolates were randomly selected from the *Campylobacter jejuni* pool isolated from the chicken samples. Their resistant and sensitive profiles are established in the results as indicated by the PCR assay.

For the abattoir samples, 12 isolates were randomly selected from various processing steps and their antibiotic biotypes determined as per PCR as indicated in the results section.

Table 3.6: List of Given Resistant Clinical Isolates (obtained from the Red Cross Children's Hospital)

Isolate Designation and origin	Strain Biotype
MF 1201 from stool	<i>C. jejuni I</i>
MF 132 from stool	<i>C. jejuni I</i>
105 from stool	<i>C. jejuni I</i>
91.01 from stool	<i>C. jejuni I</i>
117 from stool	<i>C. jejuni I</i>
MF 1238 from stool	<i>C. jejuni I</i>
MF 125 from stool	<i>C. jejuni II</i>
133 from stool	<i>C. jejuni II</i>
144 from stool	<i>C. jejuni I</i>
143 from stool	<i>C. jejuni I</i>
136 from stool	<i>C. jejuni I</i>
141 from blood culture	<i>C. jejuni I</i>
142 from blood culture	<i>C. jejuni I</i>
140 from blood culture	<i>C. jejuni II</i>
147 from blood culture	<i>C. jejuni II</i>
150 from stool	<i>C. jejuni I</i>

Isolate Designation and origin	Strain Biotype
838/ D1 from stool	<i>C. jejuni I</i>
838/ D2 from stool	<i>C. jejuni I</i>
970/ C1 from blood culture	<i>C. jejuni I</i>
970/ C2 from blood culture	<i>C. jejuni II</i>
153 from stool	<i>C. jejuni I</i>
156 from stool	<i>C. jejuni I</i>
154 from stool	<i>C. jejuni I</i>
157 from stool	<i>C. jejuni I</i>
158 from stool	<i>C. jejuni I</i>
127 from stool	<i>C.coli</i> (negative control)

Table 3.7: List of Given Sensitive Clinical Isolates (obtained from the Red Cross Children's Hospital)

Isolate Designation and Origin	Strain Biotype
108.01 from stool	<i>C. jejuni I</i>
103.01 from stool	<i>C. jejuni I</i>
88.01 from stool	<i>C.jejuni I</i>
1003 from stool	<i>C. jejuni II</i>
15 from blood culture	<i>C. jejuni I</i>
1134.2 from stool	<i>C.jejuni II</i>
11847 from stool	<i>C. jejuni II</i>
123 from stool	<i>C.jejuni I</i>
120 from stool	<i>C.jejuni I</i>
118 from stool	<i>C.jejuni I</i>

133 from stool	<i>C.jejuni II</i>
134 from stool	<i>C.jejuni II</i>
39 from blood culture	<i>C.jejuni I</i>
32 from blood culture	<i>C.jejuni I</i>
128 from stool	C.coli (negative control)

Table 3.8: List of Poultry Isolates and their Antibiotic Biotypes as per PCR Testing with Primers A1, A2 and A3 (Refer to figure3.3)

Isolate Designation	Specie Type	Antibiotc Biotype (Resistant or Sensitive)
28.3	<i>C. jejuni</i>	R
23	<i>C. jejuni</i>	R
25.1	<i>C. jejuni</i>	R
30.1	<i>C. jejuni</i>	not visible
22	<i>C. jejuni</i>	R
31.1	<i>C. jejuni</i>	not visible
30.2	<i>C. jejuni</i>	not visible
24	<i>C. jejuni</i>	R
26	<i>C. jejuni</i>	R
20	<i>C. jejuni</i>	not visible
25	<i>C. jejuni</i>	R
32.1	<i>C. jejuni</i>	R
8	<i>C. jejuni</i>	not visible
20.1	<i>C. jejuni</i>	R
7	<i>C. jejuni</i>	R

Isolate Designation	Specie Type	Antibioc Biotpe (Resistant or Sensitive)
31.2	<i>C. jejuni</i>	R

Table 3.9: List of Abattoir Isolates and their Origin of Isolation along the Processing steps

Isolate Designation	Specie type	Processing steps
Ble 1c	<i>C. jejuni</i>	Bleeding
Ble 2c	<i>C. jejuni</i>	Bleeding
Ble 3c	<i>C. jejuni</i>	Bleeding
Sca 1c	<i>C. jejuni</i>	Scalding
Sca 2c	<i>C. jejuni</i>	Scalding
Sca 3c	<i>C. jejuni</i>	Scalding
Plu 1c	<i>C. jejuni</i>	Plucking
Plu 2c	<i>C. jejuni</i>	Plucking
Evi 1c	<i>C. jejuni</i>	Evisceration
CH 1c	<i>C. jejuni</i>	Chilling
CH 2c	<i>C. jejuni</i>	Chilling
CH 3c	<i>C. jejuni</i>	Chilling

3.4 Results

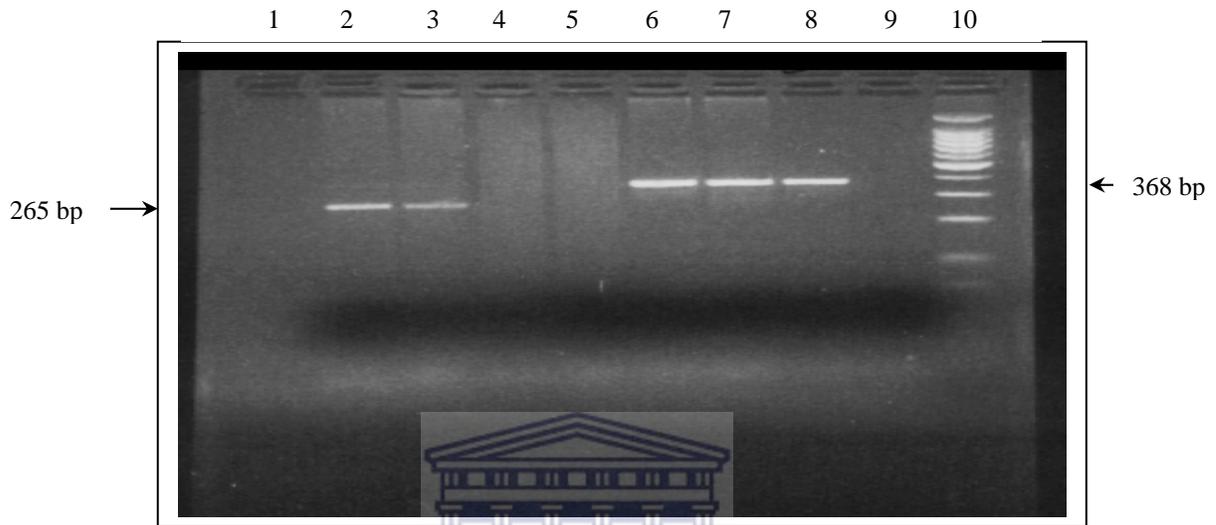


Figure 3.1: PCR profile of Quinolone resistant and sensitive isolates of *Campylobacter jejuni* by individual primer sets

Lane 10 = 100 bp DNA ladder , Lane 2&3 = quinolone resistant isolates (primers A2 & A3), Lane 6,7 and 8 = quinolone sensitive isolates (primers A1 & A2)

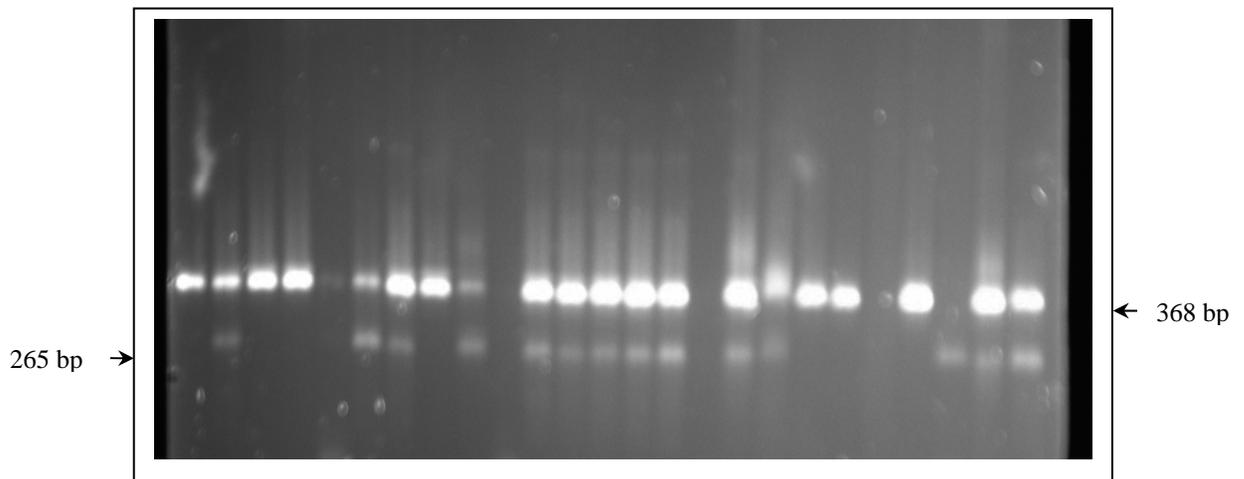


Figure 3.2: PCR profile of Quinolone resistant and sensitive clinical isolates of *Campylobacter jejuni* by multiplex PCR

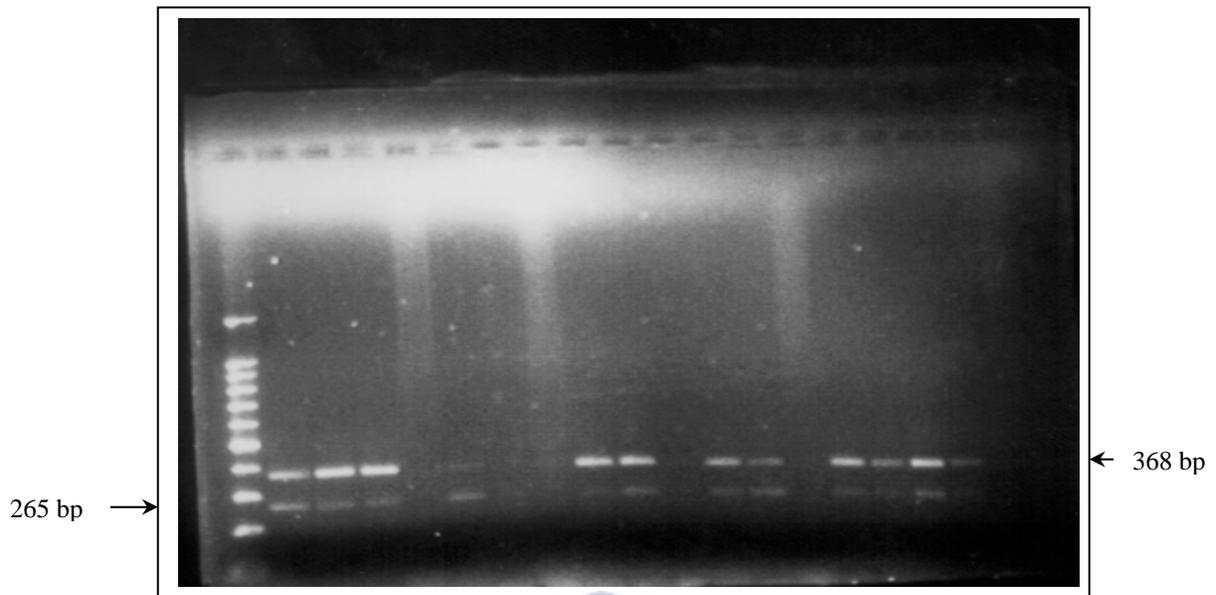


Figure 3.3: PCR profile of Quinolone resistant and sensitive poultry isolates of *Campylobacter jejuni* by multiplex PCR

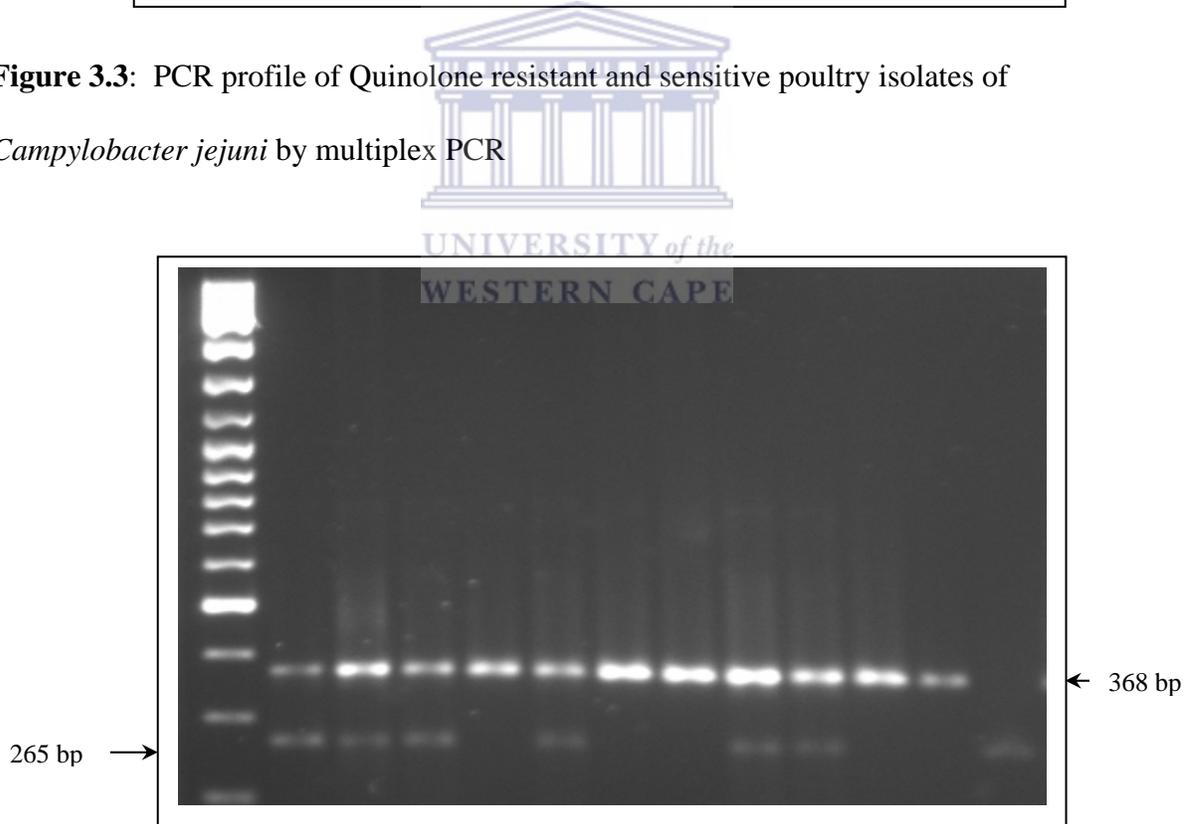


Figure 3.4: PCR profile of Quinolone resistant and sensitive abattoir isolates of *Campylobacter jejuni* by multiplex PCR

3.5 Discussion and Conclusion

The threonine to isoleucine mutation, a single point mutation has been observed according to many literatures (Charvalos *et al.* 1996, Gaunt *et al.* 1996, Ruiz *et al.* 1998, Wang *et al.* 1993) as the single change needed to confer resistance to quinolones or fluoroquinolones in *Campylobacter jejuni*. With the design of a primer set to detect this point mutation, one would thus be able to distinguish between sensitive or wildtype and resistant isolates of *Campylobacter jejuni*, as sensitive isolates does not contain this mutation. Hence only mutants are amplified signifying the presence of resistant isolates.

This was the basis for this study; to find a simple, rapid and effective way to differentiate between resistant and sensitive isolates of *Campylobacter jejuni* without the need for antibiotic susceptibility tests and the need for sequencing. The PCR procedure for detecting the mutation has proved to be successful amongst isolates in the United States where these tests have been initiated (Zirnstein *et al.* 1999). However, the South African isolates employed in this study too have had a one hundred percent success rate. Not one discrepancy has been observed. All previously identified isolates in terms of their antibiotic biotypes showed results in correlation with that expected from the PCR profiles. Sensitive or wildtype isolates yielded a single amplicon at 368 bp whilst resistant isolates yielded two products, one at 368 bp and the other at 265 bp amplified by the reverse mutation detection primer A3 and conserved forward primer A2.

The mutant PCR product (band at 265 bp) is recessive in the multiplex PCR assay and as such does not produce a highly visible band (as per figures 3.2, 3.3 and 3.4).

Another possibility is that the mutant DNA collectively may be of lower concentration than the collective wildtype isolate DNA and as a result of the ethidium bromide intercalating between bases, a lighter band is produced. Thirdly, optimal temperature preferences for each primer used in the multiplex assay may vary slightly and hence not show equally bright bands. However, individual PCR assays with only single primer sets (either primers A1 and A2 or Primers A2 and A3) will yield highly visible bands (as per figure 3.1) since there is no competition for reagents amongst primers. But these individual PCR runs do have its disadvantages. Conserved forward and reverse primers A1 and A2 will amplify a product (368 bp) for any *C. jejuni* isolate, whether resistant or sensitive. Their difference is only established when primers A2 and A3 are used in a new run for the same samples, resulting in the absence of a band for all sensitive isolates, and the presence of a band (265 bp) for all resistant isolates. This procedure is therefore time consuming, labour intensive and prone to human error. The multiplex PCR procedure is thus a fast and reliable means of directly differentiating between resistant and sensitive isolates in only one single step.

Of the 16 randomly selected poultry isolates, 12 isolates clearly produced bands, all of which were quinolone resistant (as per figure 3.3). The poultry isolates were obtained from a highly reputable chicken farm which chose to remain anonymous. The fact that resistant isolates are present is indicative that somehow resistant organisms are being transmitted from external factors. It is possible that they could be picked up from the drinking water, from the feed, from other birds or even from the hands and boots of workers. The drinking water has previously been tested by a colleague at the University of the Western Cape,

which successfully isolated *Campylobacter jejuni* and *Campylobacter coli*. This could be as a result of contamination of the water by the beaks of the birds. Also, the feed constituents are often not a true reflection of the real feeds fed to birds. The use of any antibiotics in animal feeds goes unreported for protection purposes and as such researchers find it difficult to speculate where exactly the route of transmission begins.

As figure 3.4 suggests, 9 of the 11 isolates obtained from the abattoir samples are all resistant. These isolates were obtained from various steps along the processing plant. These steps included bleeding, scalding, plucking, evisceration, and finally chilling in that order. After processing, the chickens are packaged and distributed onto shelves ready for retail. It is therefore evident that chickens that are ready for retail are not only contaminated with *Campylobacter jejuni* but with already resistant organisms.

Although finding the link between serotypes isolated from foods and those serotypes implicated in food poisoning lies beyond the scope of this study, common sense tells us that some isolates present in foods ready for retail will ultimately end up causing disease in some people. With the lack of education on food safety tips and preparation of foods that make them 'safe to eat', consumers are often unaware of the potential hazards available in certain foods, especially poultry. Hence, food producers should go out of their way to ensure that foods are completely free of hazards before its distribution onto shelves. In this way any chance of food poisoning through negligence of the cook can be severely lessened. The fact that resistant bugs are present in poultry is another cause for concern. Government should discourage the use of antimicrobials in animal feeds and should ban all such activities if possible. Farmers should be made aware of the antimicrobial resistance

phenomenon and its rapid rise through misuse of crucial drugs needed for curing disease and not for getting cattle and chickens fat faster.

This research has thus yielded a two-fold result. Firstly, with the use of this PCR assay, the presence of the mutation relevant to fluoroquinolone resistance has been determined. However, although the majority of fluoroquinolone resistant *C. jejuni* isolates have the threonine to isoleucine mutation at codon 86 of the gyrase A gene, there may be other additional mutations available that could also result in or enhance the level of resistance to quinolones. In this sense, sequencing would be suggested to detect any other mutations in the gyrase A gene after which their significance could be determined. Secondly, the resistant bugs isolated from poultry too carried the mutation significant in fluoroquinolone resistance. This PCR assay was therefore a quick screening method for detecting fluoroquinolone resistant *Campylobacter jejuni* isolates in poultry samples and can be used by public health laboratories interested in quickly characterizing the resistance profiles of isolates implicated in outbreaks. If fluoroquinolone resistant *C. jejuni* isolates test positive by antimicrobial susceptibility tests but negative by this PCR assay, then further sequencing of the gyrase A, gyrase B, par C and par E genes will be suggested (Drlica *et al.* 1997). Other resistance mechanisms, such as efflux pumps, permeability factors, or detoxification, could be studied if no other obvious mechanisms appear to be causing fluoroquinolone resistance in the isolates being examined (Drlica *et al.* 1997). This PCR assay is therefore a rapid and inexpensive means of determining whether the Thr-86-to-Ile mutation, the mutation most frequently implicated in fluoroquinolone resistance, is responsible too for the resistant *Campylobacter jejuni* organisms isolated around the Cape Town area.

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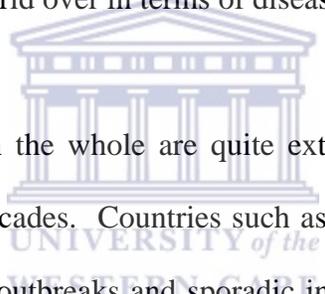
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CHAPTER 4

CONCLUSION

Worldwide reports, that is both from industrialised and developing countries are documenting *Campylobacter jejuni* to be the most important bacterial agent responsible for gastrointestinal infections and diseases (Engberg *et al.* 2004). To date, no other bacterial genus, specie or strain has yet exceeded the potential harm that *C. jejuni* has already manifested on individuals the world over in terms of disease.



Literature on *Campylobacter* on the whole are quite extensive and have been described quite thoroughly for past two decades. Countries such as the United States, Japan and all over Europe have most of their outbreaks and sporadic incidences of *Campylobacter* well documented due to the availability of good reporting systems in place. Unfortunately, in South Africa, fatalities and mortalities due to diseases often go unreported and as such much important information is lost. It therefore becomes difficult to substantiate exactly how many individuals are affected by gastroenteritis in this country each year. Nevertheless, individuals that are highly impoverished, and with obvious weakened immune systems are more susceptible to gastrointestinal infections. Based on this characteristic and the fact that a large part of South Africa's population is impoverished, it would not be surprising if *Campylobacter*, and even infections caused by other enteric pathogens such as *Salmonella* and *Escherichia coli* were to be high.

Raw poultry meat has often been suggested as the major source for human *Campylobacteriosis* because of the frequent occurrence in poultry meat at the retail level and the fact that several studies conducted worldwide have repeatedly identified handling of raw poultry and eating poultry products as important risk factors for sporadic *campylobacteriosis* (Studahl and Andersson, 2000; Effler *et al.* 2001; Kapperud *et al.* 2003; Neimann *et al.* 2003; Friedman *et al.* 2004). Another less frequent cause of an infection may be due to consumption of poorly cooked red meat, or pork, and contaminated drinking water. Based on this information it is evident that good common sense, together with proper handling and cooking of food can almost entirely eliminate the risk from *C. jejuni* infections. High enough temperatures (such as 60°C) for a short period of time (about 5 minutes) are sufficient to kill off most of the *Campylobacter* present in the food. Furthermore, air drying and overnight refrigeration will further reduce the number of organisms that may still be present. Yet, the risk of contracting *Campylobacter* infections within the kitchen environment is still high and can only be reduced by the help of the processing plants that deliver foods to the shelf ready for retail.

Processing plants may be an important entry point for *C. jejuni* into the food supply (Anderson *et al.* 2001). Here, programs such as the Hazard analysis and critical control point (HACCP) can help to identify just where in the food production process are foodborne organisms introduced or spread during production. Implementation of HACCP programs in poultry should significantly reduce levels of both *Campylobacter* and *Salmonella* as previously been documented by the US Department of Health and Human Sciences in 1999.

The risk to human health by *Campylobacter* infections are aggravated if the infection is caused by resistant organisms. An important source of resistant *Campylobacter jejuni* is the farm environment due to the use of antimicrobials and antibiotics in animal feeds. Antimicrobials are added to the feed or drinking water of poultry and farm animals to aid as a prophylaxis and to promote growth. This may all be well and good for the farmer and animal but may be disastrous to the individual who consumes that food and becomes ill. Humans pick up *Campylobacter* through the food chain. Resistant organisms are obviously more pathogenic and harder to destroy, resulting in a longer duration of illness in a person, and possibly a development of more serious complications (Engberg *et al.* 2004; Nelson *et al.* 2004). Government agencies should have a clearer regulatory authority at the farm level so as to monitor and provide an important check on the prevalence of resistance. The most obvious way would firstly be to ban the use of important antimicrobials in veterinary medicine, thereby finding other ways to treat animal enteric infections. Microorganisms have the ability to overcome the effects of antibiotics and antimicrobials in as easy as a single mutational change in their DNA, or even just structural changes in their cell membranes. The ease with which sensitive microbes are able to revert to resistant forms, sometimes even multi-resistant forms, will ultimately present challenges in the health department much greater than may be anticipated. It is already evident that humans are losing the battle with the resistance phenomena in microbes and if steps are not taken to dampen the rapid rise of resistance in microorganisms, especially within the food chain, then only humans would stand to pay the expensive price.

Due to the lack of antimicrobial resistance monitoring in South Africa, this study was aimed at investigating the possible presence of resistant isolates of *Campylobacter jejuni* in poultry samples on the farm and poultry samples after processing and prior to retail. The

choice of antimicrobial tested was a fluoroquinolone, because it was the most recently developed drug, and approved antimicrobial for use in veterinary medicine. The farm from which the samples used in this study was obtained, provided no data or evidence whatsoever of their using these drugs in their poultry growth and maintenance programs. However, the results obtained through the use of probes targeting specific mutations involved in the mechanism of fluoroquinolone resistance in a simple polymerase chain reaction procedure, enabled us to identify the presence of resistant organisms in these poultry samples.

Any bacterial organism will only become resistant to a particular drug if it had previous exposure to that drug. Evidently, fluoroquinolones are fed to chickens on that farm in one way or another and as such could present the potential risk of humans picking up these resistant organisms by means of the food chain. The possibility of investigating the link between consumption of contaminated poultry meat (especially with resistant *Campylobacter jejuni*) and disease in humans through strain comparison studies would present an interesting study for awareness and future decision making, especially in South Africa.

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APPENDIX I

MEDIA AND REAGENTS FOR BIOCHEMICAL TESTS

1. Hippurate Hydrolysis Test – only *C. jejuni* subspecies are positive.

- Stock = 5% hippuric acid (sodium salt; Sigma H 9380; Merck 820648) in distilled water
- Hippurate broth - hippuric acid stock solution 25 ml
- distilled water 100 ml
- Filter and tube in about 1 ml amounts in glass tubes. Do not autoclave.
- Ninhydrin Solution – store in deep freeze
 - - Ninhydrin (Merck 6762, BDH 10132 4E) 3.5g
 - - 50:50 Butanol in Acetone 100 ml

1.1 Thaw hippurate broth and inoculate heavily.

1.2 Incubate overnight (more practical than 2 hours).

1.3 Gently add about 0.5 ml ninhydrin solution. Do not shake (aerate) the tube.

1.4 Read within 10 minutes (2 hour test needs 30 minutes incubation) for following

results:

Positive = purple

Negative = colourless or light purple

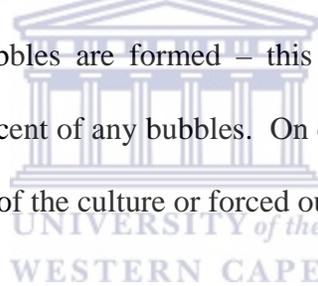
Hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidizing agent, ninhydrin. Ninhydrin becomes reduced in the process and the end products of the ninhydrin oxidation form a purple-coloured dye. (Sometimes H₂ cultures may give false positive results – repeat from CO₂ growth).

2. Catalase Test:

A loopful of culture (taken carefully from a TBA plate) is picked up with a capillary tube containing hydrogen peroxide (20 volumes). Oxygen bubbles are trapped in the capillary tube.

C. jejuni = positive

Catalase breaks down the hydrogen peroxide into oxygen and water. A positive reaction can be so strong that large bubbles are formed – this can very successfully block the capillary tube and prevent the ascent of any bubbles. On close scrutiny the trapped bubbles can be seen stuck on the surface of the culture or forced out at the bottom.



3. Tryptose Blood Agar

Tryptose blood agar		30g
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H ₂ O	to	1L
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Autoclave, cool and add 10% horse blood

APPENDIX II

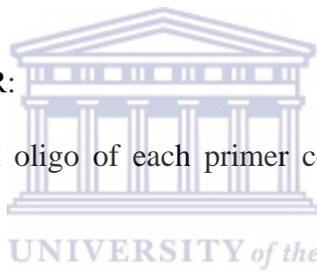
Media, Reagents and Solutions for Molecular Tests

Sodium Dodecyl Sulphate (SDS) – 10%

Dissolve 10g of SDS in 90ml of sterile water. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCL. Adjust the volume to 100ml with water.

1. Primer concentrations for PCR:

1.1. Firstly calculate the μM oligo of each primer concerned by using the following formula:


$$\text{UM oligo} = \text{O.D260} \times \text{Factor (ss)} \times 1000 / \text{Length (primer y)} \times \text{DA (SS)}$$
$$=?\mu\text{M of primer y}$$

where O.D260 is given on the primer information sheet from the manufacturers (Integrated DNA Technologies, Inc., available through Promega).

Factor (ss) = 37;

Length of primer = no. of nucleotide bases of primer;

And DA (ss) = 324.5.

1.2. Make firstly 100 μl of a working stock solution (stock 1) of 50 μM for each primer using the following formula:

$$C1V1 = C2V2$$

Where, $C_1 = 50\mu\text{M}$

$C_2 =$ answer obtained from step above

$V_1 = 100\mu\text{l}$ and

$V_2 = ? =$ the volume of primer y. This volume is added to a volume of sterile water to obtain a final volume of $100\mu\text{l}$. From this working stock solution, make a $100\mu\text{l}$ of a final stock solution (stock 2) of $5\mu\text{M}$ required for the PCR as follows:

$$(5\mu\text{l} / 50\mu\text{l}) \times 100\mu\text{l} = 10\mu\text{l of stock 1} + 90\mu\text{l of sterile water.}$$

Store all reagents in the freezer and use only stock 2 of $5\mu\text{M}$ for the PCR assays.

2. dNTP's concentrations:

dNTP's (obtained from Boehringer Mannheim) of each type (dATP, dCTP, dGTP, dTTP) are required as 2.5mM concentrations in a PCR assay. Each dNTP are provided in 100mM concentrations. Hence calculate as follows for a $100\mu\text{l}$ mix of all four dNTP's:

$$(2.5\text{mM}/100\text{mM}) \times 100\mu\text{l} = 2.5\mu\text{l of each dNTP};$$

$$\text{hence } 2.5\mu\text{l} \times 4 = 10\mu\text{l of mix} + 90\mu\text{l sterile water.}$$

3. To use $1\mu\text{l}$ of Taq polymerase (obtained from Promega) in a reaction mix, first dilute the taq from $5\text{U}/\mu\text{l}$ to $1\text{U}/\mu\text{l}$. To make a $10\mu\text{l}$ stock, prepare as follows:

$$(1/5) \times 10\mu\text{l} = 2\mu\text{l Taq} + 8\mu\text{l of } 1 \times \text{reaction buffer.}$$

Alternatively, use $0.1\mu\text{l}$ of undiluted taq in a PCR reaction mix.

4. MgCl_2 Concentration:

An initial concentration of 25mM MgCl_2 is provided. PCR assays work best at a specific MgCl_2 concentration. To obtain the most optimum MgCl_2 concentration, set up various tubes of reaction mixes for the PCR keeping all reagents the same in each tube but

increasing only the MgCl₂ concentration by a set volume for each tube. For example, start with a 0 concentration of MgCl₂, with increments of 3ul MgCl₂ for each tube thereafter.

5. 3M Potassium Acetate

KAc 29.5g

H₂O 100ml

Mix and set pH to 4.5

6. 10mg/ml Proteinase K 10g

1 x TE 1ml

Mix and store in 4⁰C

7. 10mg/ml Lysozyme

Lysozyme 10g

H₂O 1ml

Mix well and store in 4⁰C

8. 70% Ethanol

100 EtOH 70ml

H₂O 30ml

Mix well and store in 4⁰C

9. 10xTBE Buffer (1L)

108g Tris

55g Boric Acid



9.4g EDTA

in 1000ml water

10. 1xTE Buffer

1M Tris – HCl (pH.8) 100ul

0.5M EDTA (pH8) 20ul

in 10ml water

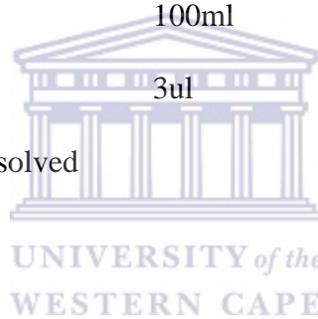
11. 2% Agarose Gel

Agarose 2g

0.5xTBE 100ml

EtBR 3ul

Mix and microwave until dissolved





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