

**A survey of post-evisceration contamination of broiler carcasses
and ready-to-sell livers and intestines (mala) with *Campylobacter
jejuni* and *Campylobacter coli* in a high throughput South
African poultry abattoir**

by

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SUMMARY

The reported incidence of human campylobacteriosis has markedly increased in developed countries within the last 20 years. The prevalence and importance of *Campylobacter* spp. as the cause of human gastroenteritis in developing countries is not known, as information is limited due to a lack of national surveillance programmes in these countries. However, it seems likely that the rate of campylobacteriosis is high among infants and children below 2 years of age resulting in substantial morbidity and, to a lesser extent, mortality.

The aim of this study was to determine the extent of contamination and cross-contamination of poultry products with *Campylobacter* in a high-throughput South African chicken processing plant. It is the first research project for the evaluation of the zoonotic risk of *Campylobacter* for consumers in South Africa. While conventional culture-based detection methods of *Campylobacter* spp. usually need 4-6 days to produce a result, the polymerase chain reaction (PCR) method developed for this research project took less than 32 hours. Both strains, *C. jejuni* and *C. coli*, are the subject of this paper and will be collectively referred to as *Campylobacter* unless otherwise stated.

During the winter of 2004, 300 samples were randomly taken from 50 chicken carcasses directly after evisceration, as well as 25 samples from ready-to-sell packages of fresh intestines (mala) and livers. The samples were taken in batches over a time period of 4 weeks. All samples were examined by means of DNA extraction and PCR resulting in the following findings: The average contamination rates with *Campylobacter* for both the skin samples and livers were 24%, and for intestines a contamination rate of 28% was found. These results are in line with the findings of other authors.

Chicken and chicken products, especially livers and intestines form an important part of the traditional diet and reflect the special African situation. They are cheap and easily available outside supermarkets and other retail outlets. Street vendors and hawkers who do not have cooling facilities or access to and washing facilities sell the products. The break in the cold chain, especially under South African climatic conditions, favours the multiplication and consequently the increase of numbers of *Campylobacter* bacteria

already present in the products. The handling of such contaminated products in households and the potential for cross-contamination of other foods presents a high risk of infection to consumers.

This research project concludes that *Campylobacter* is prevalent in poultry in South Africa and that the contamination of poultry meat and products with this organism could represent a health hazard for consumers in South Africa. It also emphasises the need for further research in this field.

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List of Abbreviations

ASC	Acidified sodium chlorite
bp	base pair
CCA	Campy-Cefex agar
CFU	Colony forming unit
GBS	Guillain-Barré syndrome
HACCP	Hazard Analysis Critical Control Point
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MHBA	Mueller – Hinton blood agar
nm	Nanometer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ppm	Parts per million
ReA	Reactive arthritis
SPF	Specific pathogen free
TAE	Tris-Acidic acid – EDTA
VBNC	Viable but non-culturable

CHAPTER 1

Introduction and literature review

1 Introduction

Campylobacteriosis in humans is the leading cause of acute bacterial diarrhoea in many countries (Alter *et al.*, 2005). *Campylobacter* enteritis is considered as important as or even more important than infections caused by *Salmonella* spp. and *Shigella* spp. in frequency of isolation (Blaser *et al.*, 1983; Griffiths and Park, 1990; Kemp and Schneider 2002). Most infections are sporadic and self-limiting and spectacular large outbreaks, severe illness and death are rare (Bryan and Doyle, 1995; Ring and Atanassova, 1999; Rosenquist *et al.*, 2003). Due to the direct and indirect costs the disease causes, however, the impact that it has on the society can be enormous (Griffiths and Park, 1990; Skirrow, 1990; Skirrow, 1991; Bryan and Doyle, 1995; Bouwknecht *et al.*, 2004).

Most cases of human campylobacteriosis are caused by *Campylobacter jejuni*. *Campylobacter coli*, *C. lari* and *C. upsaliensis* are also recognized as causing human gastroenteritis, but less frequently. While *C. jejuni* is implicated in approximately 85-99% of the cases of human campylobacteriosis in developed and developing countries, the majority of the remaining cases are caused by *C. coli* in developed countries (Le Roux and Lastovica, 1998; Smith, 2002; Anderson *et al.*, 2003; Rosenquist *et al.*, 2003; www.FoodProductionDaily.com, 2004; Alter *et al.*, 2005). In developing countries, strains like *C. upsaliensis* and *C. lari* are causing infections in humans to a higher extent than in developed countries (Anderson *et al.*, 2003).

Both *C. jejuni* and *C. coli* are the subject of this paper and will be collectively referred to as *Campylobacter* unless otherwise stated. Evidence of association between *Campylobacter* in chicken and sporadic human infection is provided by the occurrence of similar serotypes in chicken and humans and similar patterns of antibiotic resistance in chicken and humans. (Shanker *et al.*, 1982; Juven and Rogol, 1986; Moore and Elisha, 1997; Nicol and Wright, 1997; Jacobs-Reitsma and Bolder, 1998; Smith *et al.*, 1999; Pearson *et al.*, 2000).

Enteric campylobacteriosis is a typical zoonosis, which can be transmitted by direct contact with contaminated animals or animal carcasses, or indirectly by ingestion of contaminated food or water. *Campylobacter* are enteric commensals or occasional pathogens in a wide range of animals, which thus form the source of infection for

humans. *Campylobacter* can often be isolated from the faeces of dogs and cats with isolation rates higher in young than in mature animals. Infected pets form a reservoir of infection especially for children (Blaser *et al.*, 1983; Skirrow, 1990 and 1991). Carrier-animals like poultry, cattle, sheep and pigs are sources for food-borne illnesses rather than for contact infections (Blaser *et al.*, 1983; Skirrow, 1990 and 1991; Rosenquist *et al.*, 2003; Wong *et al.*, 2004). Faecal contamination of carcasses from the intestinal contents during slaughtering process and contamination of milk are incriminated as the main routes for food-borne infection of consumers (Blaser *et al.*, 1983; Joseph *et al.*, 1989; Sinell, 1985; Thurm and Dinger, 1998).

Campylobacter jejuni/coli are distributed worldwide. They are enteric commensals or occasional pathogens in numerous mammalian and avian species and in environmental waters contaminated with their faeces. There is a certain host preference, with *Campylobacter jejuni* found mainly in poultry and cattle, and *C. coli* more prevalent in pigs (Penner, 1995).

Campylobacter are especially common in wild and domestic birds. As the optimum growth temperature of *Campylobacter* is 42-43°C, birds offer the optimal environment for the bacteria due to their higher body temperature compared to mammals (Skirrow, 1990). Poultry meat is cited as the most important source of human campylobacteriosis because most commercially raised poultry harbour *Campylobacter* in their intestinal flora and contamination of carcasses and products is common during slaughtering and processing (Beery *et al.*, 1988; Bryan and Doyle, 1995; Whyte *et al.*, 2001; Rosenquist *et al.*, 2003; Wong *et al.*, 2004).

Contamination of poultry is thought to be nearly universal and colonization of birds in a flock can be detected from the second and third week of age. *Campylobacter* are usually introduced into a flock by single birds and horizontal transmission throughout the remainder of the flock is rapid. The usual infection rate in a flock is 100% (Jacobs-Reitsma, 1997; Anderson *et al.*, 2003). The large numbers of intestinal *Campylobacter* that are brought into the processing plant with the birds result in a massive contamination of birds, processing lines, equipment, hands of workers and finally the end-products. Contamination of carcasses and meat is mainly superficial or subcutaneous, and the incidence of bacteria in muscles is very low (Thomas and McMeekin, 1980). The parts of carcasses and the end products mainly contaminated are the peritoneal cavity, breasts, thighs and drums. Numbers of organisms can exceed 10⁶/g (Skirrow, 1991).

Unlike *Salmonella*, *Campylobacter* do not multiply in food, but as the infection dose for humans is low, just a few bacteria (400 – 500) are necessary to cause an infection.

Campylobacteriosis often results from a lack of kitchen hygiene when handling raw chicken or chicken products, from cross-contamination of ready-to-eat foods and from eating undercooked chicken (Blaser *et al.*, 1983; Oosterom *et al.*, 1983, Joseph *et al.*, 1989; Griffiths and Park, 1990; Kwiatek *et al.*, 1990; Berndtson *et al.*, 1992; Jacobs-Reitsma and Bolder, 1998).

Conventional detection of *Campylobacter* in food depends on selective cultural enrichment followed by isolation from selective agar. Identification and confirmation is based on biochemical tests. These methods are time consuming and laborious, requiring an average time of 4-6 days. DNA hybridization and polymerase chain reaction (PCR) have been developed as a rapid, sensitive and reliable alternative to detect *Campylobacter* in food samples. Several PCR assays with and without pre-enrichment have been described in the literature and a comparison study of conventional methods and PCR-based assays revealed the higher sensitivity and detection rate of the latter method (Giesendorf *et al.*, 1992; Hazeleger *et al.*, 1994; Winters and Slavik, 1995, Docherty *et al.*, 1996; Ng *et al.*, 1997; Ring and Atanassova, 1999; Waage *et al.*, 1999; Thunberg *et al.*, 2000).

1.1 Aims of this study

Since the importance of *Campylobacter* as a cause for acute gastroenteritis in man was recognized 20 years ago, tremendous research has been done on this subject in many parts of the world including European and Asian countries, North America and Australia.

However, the literature review has shown a paucity of information on the current situation in South Africa especially regarding the prevalence of *Campylobacter* in the poultry industry and its importance as a food borne zoonosis. The limited studies performed on *Campylobacter* in southern Africa and the reports about the prevalence and epidemiology of the pathogen in other developing countries indicate a strong need for investigation in South Africa. Similar findings as cited in the literature review are expected as an outcome of this study.

The main objectives of this study were:

1. To determine the extent of contamination and cross-contamination of poultry products in one high throughput South African chicken processing plant
2. To develop a convenient and practical method for identifying *Campylobacter jejuni* and *Campylobacter coli* in the obtained samples.

2 *Campylobacter* spp.: The agent

Campylobacter species are small, slender, curved, Gram-negative rods (1.5 – 5 µm long, 0.2 – 0.5 µm wide). They are S-shaped and often two or more organisms are joined at their ends to form a spiral chain. *Campylobacter* are motile by a single polar unsheathed flagellum at one or both sides of the cells. They show a characteristic, rapid corkscrew-like motion. *Campylobacter jejuni* is able to move rapidly in a viscous environment such as it is provided by intestinal mucus (Mayr, 1984).

Campylobacter spp. are microaerophilic and an oxygen concentration of 5-10% has been determined to be optimal for growth. They are oxidase-positive, have a respiratory-like metabolism and do not ferment or oxidize carbohydrates. The tests for catalase and H₂S production, nitrate reduction, hippurate hydrolysis, and susceptibility to nalidixic acid and cephalotin are used for identification. Growth temperatures vary widely with respect to optimum and range, but all species grow at 37°C. The growth optimum of *Campylobacter jejuni* and *C. coli* is at 42°C, but they do not grow below 30°C. Therefore they are often referred to as thermophilic *Campylobacter* (Griffiths and Park, 1990; Fraser *et al.*, 1991; Quinn *et al.*, 1994; Hunt *et al.*, 1998).

When environmental conditions are unfavourable, *Campylobacter* cells transform very quickly from the spiral form into a coccoid form. These cells are viable but non-culturable (VBNC) and thus difficult to detect with culture-based methods. While some researchers consider the VBNC state of *Campylobacter* as a degenerative form, others rate the infectivity of the coccoid form similar to that of the spiral cell form (Archer, 1988; Hazeleger *et al.*, 1994; Diergaardt, 2001).

In a moist environment, such as on the surface of poultry, *Campylobacter jejuni/coli* can survive for several weeks at 4°C, and often outlast the shelf life of the product (except in raw milk products). They are sensitive to freezing but some cells remain viable and can be isolated after several weeks of frozen storage. Environmental stress like exposure to air, drying, low pH, heating, freezing and prolonged storage damages cells and hinders recovery to a greater degree than for most bacteria (Griffith and Park, 1990; Hunt *et al.*, 1998).

The pathogenicity of *Campylobacter jejuni* is not properly understood, but it is probably based on three (3) pathogenic factors:

- An adhesin needed to enable the organism to colonize the mucosal surfaces

- A heat-labile toxin similar to that of *Escherichia coli*, which may induce the watery diarrhoea seen in many patients with campylobacteriosis
- A cytotoxin, which is the cause of the presence of blood in the stool of some patients (Griffiths and Park, 1990; Quinn *et al.*, 1994).

3 *Campylobacter* infections in poultry

Today in most developed countries *Campylobacter* are the most frequently identified agents of acute infective diarrhoea. *Campylobacter* enteritis is caused by the two closely related species *Campylobacter jejuni* and *Campylobacter coli* with more than 100 serotypes. *Campylobacter jejuni* is the predominant species, but the distinction of the two is mainly of epidemiological interest since the disease caused by each species is the same (Skirrow, 1990).

Campylobacter spp. are widely distributed in poultry flocks including breeders, laying hens and broilers. Due to the enormous consumption of poultry meat, infected broiler flocks are by far the biggest potential health hazard for humans.

Broiler intestinal material, containing *Campylobacter* spp., can easily contaminate large numbers of broiler carcasses during slaughtering and processing. If not handled properly, contaminated end products might lead to human illness. Thus the prevention of colonisation of *Campylobacter* in broilers will add considerably to public health (Jacobs-Reitsma *et al.*, 1994).

Infection of poultry is not generally associated with clinical illness even though large numbers of *Campylobacter* are excreted in the faeces. *Campylobacter jejuni* colonizes primarily the lower gastrointestinal tract of the chicken, i.e. caeca, large intestine and cloaca. Here the bacteria are densely packed in mucus within the crypts without attachment to crypt microvilli. *Campylobacter* is chemo-attracted to mucin and able to move freely within the mucus. In addition, it can utilize mucus as a sole substrate for growth (Beery *et al.*, 1988; Evans and Sayers, 2000).

Campylobacter jejuni causes a contagious hepatitis in poultry known as 'avian vibronic hepatitis' (Avian infectious hepatitis). Subclinical infection is common in chickens, and while the clinical illness causes a reduction in egg production, morbidity and mortality is rare. The majority of infections in chickens are subclinical and confined to the intestinal tract. Clinical disease usually is chronic, with typical symptoms such as weight loss,

appearance of shrivelled, dry and scaly combs, listlessness, diarrhoea and apathy (Siegmann, 1993).

Typical pathological lesions of acute clinical disease are haemorrhagic and necrotic changes in the liver, and heart lesions. A presumptive diagnosis can be made from a typical history of clinical disease in a flock. Isolation of *Campylobacter jejuni* from bile or liver and faeces should confirm a presumptive diagnosis (Mayr, 1984; Fraser *et al.*, 1991; Siegmann, 1993).

During the last few years ostrich farming for meat has become very popular worldwide. Ostriches are now classified as poultry rather than as feathered game. The increasing consumption of ostrich meat raises concern about possible zoonotic pathogens associated with ostriches. Enteritis and hepatitis caused by *C. jejuni* have been found in young ostriches in South Africa while an Australian study revealed *C. coli* as cause of avian hepatitis in ostrich chicks. The possible zoonotic hazard of *Campylobacter* in ostriches has still to be determined (Stephens *et al.*, 1998; v. d. Walt *et al.*, 1997).

Once *Campylobacter* is evident, it spreads rapidly within the flock in animals between two and five weeks of age. The prevalence of infection is directly related to increasing age of the chickens (Evans and Sayers, 2000; Bouwknegt *et al.*, 2004). So far, no natural *Campylobacter* infection was detected in birds younger than two weeks. Colonization usually reaches up to 100% within one to two weeks and remains high up to slaughter (Jacobs-Reitsma *et al.*, 1994; Berndtson *et al.*, 1996b; Evans and Sayers, 2000). These findings will probably be very similar in South Africa. Although several studies conducted abroad show a worldwide high incidence of *Campylobacter* in poultry flocks, no published data is available regarding the frequency of infection in poultry flocks in South Africa.

No evidence of vertical transmission of *Campylobacter* has been found. The major route of *Campylobacter* colonization in a flock is horizontal transmission from the environment like drinking water, contaminated air within a flock house, dirty transport crates and rodents present on the farm. *Campylobacter* are usually introduced into a flock by only a few birds and the spreading over the whole population of a production unit/broiler house is rapid (Anderson *et al.*, 2003). Other farm animals, especially sheep, pigs and laying hens as well as rodents are often found to be permanent carriers of *Campylobacter* and can therefore be regarded as a potential source of infection for broilers. Contaminated litter does not seem to play a role in the transmission of *Campylobacter* (Bryan and Doyle, 1995; Berndtson *et al.*, 1996a; Berndtson *et al.*, 1996b; Payne *et al.*; 1999).

Good hygiene standards on farms and the use of an all-in-all-out-system with proper cleanout and disinfection between the flocks are effective measures to reduce the colonization of a flock. This will result in a reduced risk of human infection with *Campylobacter* (Hoop and Ehram, 1987, Beery *et al.*, 1988; Jacobs-Reitsma *et al.*, 1994; Jacobs-Reitsma, 1997; Saleha *et al.*, 1997, Evans and Sayers, 2000).

4 **Campylobacteriosis in humans**

4.1 **Incidence**

Campylobacter were once thought to be a microorganism of mainly veterinary concern and only sporadically causing diseases in humans, but the number of reported cases of *Campylobacter* enteritis has increased dramatically over the last 20-30 years (WHO, 2000; Anderson *et al.*, 2003; Alter *et al.*, 2005). In almost all developed countries, campylobacteriosis is now the leading cause of human gastrointestinal infections (Harris *et al.*, 1986; Doyle, 1994; WHO, 2000; Anderson *et al.*, 2003).

According to Kwiatek *et al.* (1990), the prevalence of *C. jejuni* in patients with acute gastroenteritis ranges from 2-14% in various countries. Most human cases of *Campylobacter* infections are classified as sporadic, single cases, which are attributed to the consumption of contaminated food with poultry meat being the leading cause (Beuchat, 1996; Pearson *et al.*, 2000; Wong *et al.*, 2004). Large outbreaks are rare and are usually associated with contaminated milk or surface water (Griffiths and Park, 1990; Skirrow, 1991; Thunberg *et al.*, 2000).

Since the mid 1970s, increasing research has been carried out on the role of *Campylobacter* in causing illness in humans as well as on the development of effective sampling and isolation methods. The rise in reported human cases of *Campylobacter* enteritis is therefore not only a real increase in incidence of cases but rather a sign of more concern about the organism as a human pathogen and also as a result of better methods for isolation and detection of *Campylobacter* spp. (Bryan and Doyle, 1995). In developed countries, changes in eating habits may also contribute to the rise in human *Campylobacter* infections with a larger amount of consumed poultry and an increase in consumption of “take-away” fast foods (Doyle, 1981; Griffiths and Park, 1990; BgVV, 1998).

Only little information is available regarding the prevalence of human campylobacteriosis in developing countries due to a lack of national surveillance. However, it is likely that the incidence is especially high amongst infants and young children (Doyle, 1981; Blaser *et al.*, 1983; Le Roux and Lastovica, 1998; Anderson *et al.*, 2003). A survey carried out in

Nigeria revealed that over the past decade human campylobacteriosis emerged as an important zoonosis due to climatic, ecological, agricultural and socio-economic factors having major influence on the veterinary public health and public health sectors (Coker *et al.*, 2000). A survey performed by Simango *et al.* in 1992 in Zimbabwe, revealed a low contamination of prepared food and drinking water with *Campylobacter*. DeMol and Bosmans (1978, cited by Doyle, 1981), revealed a prevalence of 10.8% of *C. jejuni* in patients with diarrhoea in Rwanda.

4.2 Gender distribution

In the literature there is no remarkable difference in the incidence of infection between genders. Only Doyle (1981) cites one report where a male to female infection rate of 3:2 was stated. A risk assessment study by Rosenquist *et al.*, 2003, in Denmark, supports these findings (Figure 1.1).

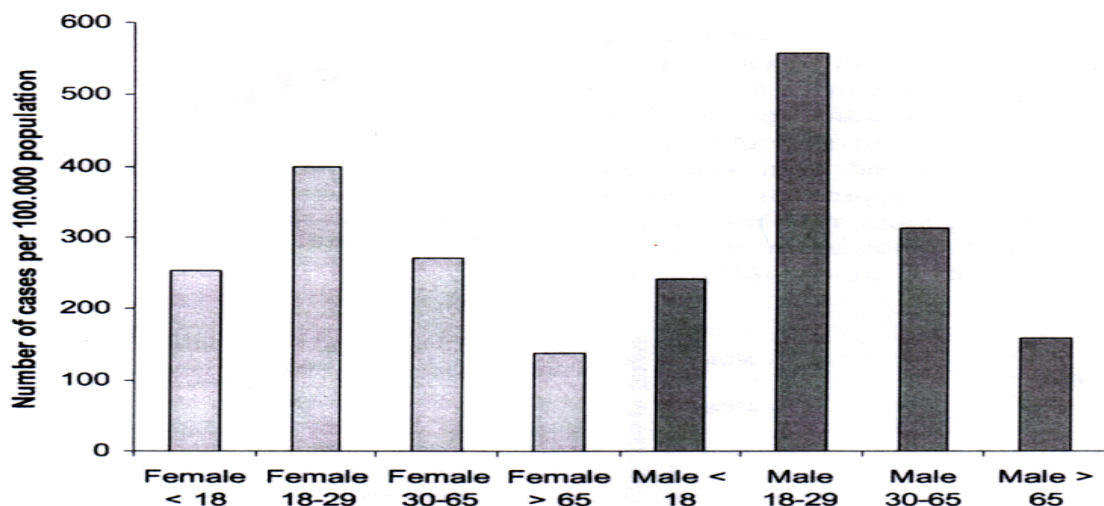


Figure 1.1 Estimated values of the incidence of campylobacteriosis associated with the consumption of a chicken meal for different age and sex groups (Rosenquist *et al.*, 2003).

4.3 Age distribution

Many early researchers did not find a difference in the incidence of infection in the various age groups in developed countries. However, Blaser *et al.* (1983) and Skirrow (1990) state a bimodal age distribution with peaks of incidence in infants under 5 years, and young adults aged between 15 and 29 years. The reasons for this pattern are unknown but it might be the popularity of fast food consumption in young adults and the fact that infants with diarrhoea are presented more often to the practitioner than the

average of affected people (Blaser *et al.*, 1983; Skirrow, 1990). Bryan and Doyle (1995) also described a bimodal distribution pattern of *Campylobacter* infections with the first peak among infants and the second peak among adults 20 to 30 years of age. These authors relate the peaks to times of the weaning phase in children and when persons set up their own housekeeping and prepare foods.

In developing countries, the vast majority of infections occur in children in the first five years of life. In children between 0 and 24 months the incidence of infection and the severity of the resulting illness is the highest (Anderson *et al.*, 2003). A survey performed in South Africa established an infection rate with *C. jejuni* of 13.4% in black children and 4.9% in Caucasian children below two years of age (Blaser *et al.*, 1983). Bokkenheuser *et al.* (1979, cited by Doyle, 1981) performed a survey in Soweto where 34% of all children with diarrhoea were positive for *C. jejuni* while the organism could be detected in 12.5% of asymptomatic children. In Shanghai, China, *C. jejuni* was found in 13% of stool samples taken from children under three years of age suffering from diarrhoea (Blaser *et al.*, 1983, citing Mauff *et al.*, 1981).

The following aspects are regarded as main causes for the high infection rates in infants: a strong environmental exposure, together with poverty, overcrowding, under nutrition, poor hygiene and dangerous bottle-feeding habits. Like other diarrhoeal diseases caused by bacteria, *Campylobacter* infections in children result in high mortality rates (Ireland, 1998). Surveys since the 1980s conducted at the Red Cross Children's Hospital in Cape Town revealed that the isolation of *Campylobacter* and related species has risen dramatically. At the same hospital a survey was carried out between October 1990 and September 1997 to determine the distribution of *Campylobacter* from stools obtained from children admitted with diarrhoea (Le Roux and Lastovica, 1998). It revealed that thermophilic *Campylobacter* such as *C. jejunicoli* were present in nearly 50% of all samples. This rate of *Campylobacter* isolation described by Le Roux and Lastovica in the "Cape Town Protocol" is unequalled anywhere (Blaser *et al.*, 1983; Ireland, 1998; Le Roux and Lastovica, 1998).

The lower rate of infections in adults in developing countries may be due to a good immunity gained in early childhood, which is thought to be the result of early exposure to the organism (Skirrow, 1990).

4.4 Geographical distribution of *Campylobacter*

Epidemiological differences affecting the age groups and the severity of illness have been observed between developed and developing countries. In developed countries, *Campylobacter* enteritis often affects older children and young adults, and the illness is

often severe requiring antimicrobial therapy. The number of cases reported is higher in developed countries than in developing countries, where children younger than one year usually show very severe cases of illness, while in older children and young adults the course of the disease is milder (Anderson *et al.*, 2003).

The incidence of infection in developing countries is much higher in rural than in urban populations. While nutritional factors are seen as the major cause for infections in the urban areas, in rural living conditions the exposure to *Campylobacter* due to environmental contamination from domestic animals is probably the most important factor for transmission (Anderson *et al.*, 2003). In developing areas where hygienic conditions are poor, the prevalence of *Campylobacter* infections amongst children is higher than in areas with good hygienic conditions (Blaser *et al.*, 1983).

Campylobacter enteritis is reported frequently from travellers returning from tropical countries and is therefore often referred to as travellers' diarrhoea (Skirrow, 1990).

5 Clinical signs / Pathogenesis

Campylobacter is the leading cause of zoonotic enteric infection in most developed and developing countries (Blaser *et al.*, 1983; Atanassova and Ring, 1999; WHO, 2000; Anderson, 2003). In almost all developed countries, the reported incidence of human campylobacter infections has been steadily increasing for several years (Griffiths and Park, 1990; WHO, 2000; Rosenquist *et al.*, 2003). However, the true rate of infection is estimated to be 7.5 up to 100 times higher than the reported figures (Anderson *et al.*, 2003).

Symptomatic *Campylobacter* infections are marked by gastrointestinal illness, which is often clinically indistinguishable from that caused by other enteric pathogens (Blaser *et al.*, 1983). Generally, *Campylobacter* enteritis is self-limiting and treatment is not necessary, but infections can lead to potentially dangerous long-term consequences like bacteraemia, meningitis, pneumonia, miscarriage, reactive arthritis (ReA) and an acute flaccid paralytic disease (Guillain-Barré syndrome: GBS) (Griffith and Park, 1990; Skirrow, 1990; Hunt *et al.*, 1998; Smith, 2002). *Campylobacter jejuni* is the inducer antecedent infection in approximately 30% of all cases of GBS, while reactive arthritis, which leads to the impaired movement of various joints occurs in approximately 2% of all *C. jejuni* enteritis cases (Nachamkin and Lastovica, 1998; Smith, 2002).

Evidence shows that immunocompromised individuals are at increased risk for *Campylobacter* infections. Patients with HIV/AIDS were found to be 39 times as likely as

immunocompetent individuals to have campylobacteriosis. Patients with HIV/AIDS and campylobacteriosis also showed an increased incidence of bacteraemia and hospitalization compared with non-infected HIV/AIDS patients. Bacteraemia is uncommon and transient in immunocompetent people while immunocompromised individuals are predisposed to *Campylobacter jejuni* induced bacteraemia and a higher mortality caused by the infection. Surveillance performed over 10 years in England and Wales revealed an incidence of 25.8% of bacteraemia in immunocompromised patients. Although pregnant women and elderly people are usually considered as immunocompromised no evidence shows predisposition of these population groups to *Campylobacter* infection (Smith, 2002).

5.1 Gastroenteritis

Campylobacter enteritis is variable in severity and infections range from asymptomatic excretion of the pathogen (25 – 50% of cases) to a very severe disease resulting in death (Smith, 2002).

The average incubation period is 3 days, but can vary from 10 hours up to 11 days (Sinell, 1985; Harris *et al.*, 1986; Skirrow, 1990; Reintjes *et al.*, 1999). Typical for *Campylobacter* enteritis is the sudden onset of symptoms starting with fever of 40°C and higher that can last for 2 days. Myalgias, chills, headache, nausea and malaise usually accompany the fever, followed by severe abdominal cramps and diarrhoea. Intensity and duration of abdominal pain is often greater than with other bacterial gastroenteritides and can easily be mistaken for acute appendicitis (Griffiths and Park, 1990; Skirrow, 1990). The diarrhoea is watery and slimy, sometimes bloody. In most cases, the diarrhoea lasts about a week and is self-limiting (Rosenquist *et al.*, 2003). According to Blaser *et al.* (1979), the occurrence of gross or occult blood in the stool of patients could be an important diagnostic feature in patients with *Campylobacter* enteritis. Patients excrete high numbers of *Campylobacter* from the start of the disease, but the excretion usually diminishes two to three weeks after recovering and chronic carriage is not known in healthy people (Skirrow, 1990). According to Blaser *et al.* (1983) asymptomatic excretion of *Campylobacter* is uncommon while Smith (2002) reports that 25 to 50% of all infected people might be asymptomatic carriers and excrete the organism.

The duration of the acute illness is generally between 2 and 7 days, but up to 20% of all cases may result in relapses, or a prolonged or severe course of disease (Blaser *et al.*, 1983). Enteric campylobacteriosis has been associated with infection of the biliary tract leading to cholecystitis, pancreatitis or obstructive hepatitis (Smith, 2002).

In the majority of cases the infection is self-limiting and does not require antibiotic therapy. Oral rehydration and electrolytic replacement is the treatment of choice in most cases. Antibiotic treatment is recommended in prolonged or severe infections accompanied by bloody stools and high fever or any complications. It is also indicated in patients at risk such as immunocompromised individuals and pregnant women as *Campylobacter* can have deleterious effects on the foetus like stillbirth, abortion, meningitis or bacteraemia in the newborn (Smith, 2002). Effective antibiotics include erythromycin and other macrolides, quinolones, tetracycline and aminoglycosides. However, an increasing resistance in *Campylobacter*, both human and animal strains, to clinically useful antibiotics and even multidrug resistance has been reported (Moore and Elisha, 1998). The development of such resistance in food-borne zoonotic pathogens may have been accelerated particularly through the use of antibiotics at low or sub therapeutic levels in animal feeds and as growth promoters. Resistance against drugs like tetracycline, quinolone and trimethoprim is of serious concern regarding public health because of cross-resistances against a variety of drugs used in human medicine, which is associated with the use of antibiotics in animal husbandry (Skirrow, 1990; Jacobs-Reitsma, 1997; Moore and Elisha, 1998; Smith *et al.*, 1999; Smith, 2002).

5.2 Guillain-Barré Syndrome (GBS)

Guillain-Barré syndrome is an acute disease of the peripheral nervous system, which is triggered by an acute infection with *Campylobacter jejuni*. It is a demyelinating disorder of the peripheral nervous system leading to weakness of the limbs, which is usually symmetrical, as well as the respiratory muscles and a loss of reflexes. The condition can become chronic and even fatal (Rosenquist *et al.*, 2003). The disease is self-limiting but it can take up to several months until partial or complete recovery is reached. GBS is considered to be an autoimmune, anti-body mediated disease. It shows two pathological main forms, which are characterized by immune-mediated attacks on the different tissue structures:

- 1) An acute inflammatory demyelinating polyneuropathy
- 2) An acute motor axonal neuropathy (less frequently).

Campylobacter jejuni is recognized as the single most identifiable agent associated with the development of GBS. Several studies on patients have shown that an infection with *Campylobacter jejuni* commonly precedes GBS and that 30% of all cases of GBS result from an initial infection with *C. jejuni* (Alios *et al.*, 1998; Nachamkin and Lastovica, 1998; Smith, 2002). A recently identified variant of the GBS is the Miller Fisher Syndrome, which is characterized by ophthalmoplegia, ataxia and areflexia (Rosenquist *et al.*, 2003).

5.3 Reactive arthritis (ReA)

Another long-term consequence of a *Campylobacter jejuni* infection is a condition referred to as reactive arthritis. This is a syndrome that is characterized by a sterile inflammation of the joints due to an infection that originates at a nonarticular site, usually the genito-urinary tract or the gastrointestinal tract. The mechanisms by which antibacterial antigen triggers the inflammation of the joints is unknown, but there is evidence of a hereditary predisposition for the development of ReA. The disease is suspected to be an autoimmune condition (Skirrow, 1990; Smith, 2002).

6 Epidemiology

As *Campylobacter* are commensals in the intestinal tract of a variety of domestic and wild animals, there are several sources and ways of infection for humans. Transmission to humans can occur via direct or indirect contact with animals, animal products or environmental contamination (Kraemer, 1992).

6.1 Animal reservoirs

Poultry and birds: The colonization rate of commercially raised poultry with *Campylobacter* is nearly universal and very high. Contamination sources for flocks are contaminated drinking water and/or feed and rodents. (Jacobs-Reitsma, 1997; Saleha *et al.*, 1997; Atanassova and Ring, 2000 a and b). The role of litter in transmission and maintenance of campylobacteriosis in flocks has been widely discussed. No evidence supporting an association between infected litter and transmission was found (Hoop and Ehram, 1987; Payne *et al.*, 1999; Evans and Sayers, 2000).

Wild birds including ducks and geese frequently excrete high numbers of *Campylobacter* thus contaminating surface water and the environment (Doyle, 1981; Sinell, 1985; Hunt *et al.*, 1998).

Table 1.1 is an extract from the literature giving an overview of the prevalence of *Campylobacter* contamination of flocks in various countries around the world.

Table 1.1 Prevalence of *Campylobacter* contamination of flocks cited in the literature.

Author	Country	Year	Percentage of contamination in flocks
Genigeorgis <i>et al.</i>	US	1986	81.8% (at slaughter time)
Hoop and Ehram	Switzerland	1987	12%
Doyle	Great Britain	1994	75%
Jacobs-Reitsma <i>et al.</i>	Netherlands	1994	82%
Berndtson <i>et al. (a)</i>	Sweden	1996	100% (at slaughter time)
Berndtson <i>et al. (b)</i>	Sweden	1996	27%
Jacobs-Reitsma	Netherlands	1997	50% (spring) 100% (summer)
Saleha <i>et al.</i>	Malaysia	1997	82.4%
Atanassova and Ring	Germany	2000a	75%
Evans and Sayers	Great Britain	2000	90% (at slaughter time)
Stern <i>et al.</i>	US	2001	87.5%
Bouwknegt <i>et al.</i>	Netherlands	2004	30%

Cattle: Healthy cattle often harbour *Campylobacter*, especially *C. jejuni*, in their intestines. Faecal contamination of carcasses has occasionally been reported, however the level of contamination is low (Doyle, 1981). The main vehicle for transmission of *Campylobacter* from cattle to humans is via unpasteurised milk. Contamination usually occurs via faeces during milking, but *Campylobacter* mastitis is less frequent (Doyle, 1981; Reintjes *et al.*, 1999).

Sheep: *C. jejuni* is an important cause of epizootic infectious abortion in sheep, and in many flocks it exists without apparent signs resulting in the occasional contamination of carcasses during slaughter. Offal like liver, kidney and heart are more likely to be contaminated and transmit the pathogen to consumers (Blaser *et al.*, 1983; Skirrow, 1990). While Skirrow (1990) only cites a contamination rate of the end product (meat) of 1.4%, a recent study to determine the prevalence of *Campylobacter* in sheep carcasses after slaughter revealed a contamination rate of 17.5%. Out of these, 64.9% were *C. jejuni* and 35.1% were *C. coli* (Zweifel *et al.*, 2004).

Pigs: *Campylobacter coli* and, to a lesser extent, *Campylobacter jejuni* are commonly found as intestinal commensals in pigs. More than 50% of commercially raised pigs excrete the organisms. Oosterom recovered *C. jejuni* from 61% of asymptomatic pigs in the Netherlands (1980, cited by Doyle, 1981). Steinhäuserová *et al.* (2002) described *Campylobacter* spp. contamination in intestinal contents in 20-40% of slaughtered pigs and on the surface of 10-15%. Pig carcasses become contaminated during slaughtering and processing, and incomplete elimination of organisms from the intestines by washing and salt treatment might result in contamination of sausages (Doyle 1981; Blaser *et al.*, 1983).

Dogs and cats: *Campylobacter* are often present in the faeces of healthy dogs and cats as well as in those with diarrhoea. Young animals are more often affected than mature ones. Close contact with infected pets is especially an infection risk for children (Blaser *et al.*, 1983; Hubbert *et al.*, 1996; Ring and Atanassova, 1999).

Other animals: Healthy rodents have been found to excrete *Campylobacter* frequently. Several authors therefore consider the existence of rodents in broiler flocks as an important risk factor in horizontal transmission to poultry (Blaser *et al.*, 1983; Berndtson *et al.*, 1996a; Hubbert *et al.*, 1996; Evans and Sayers, 2000).

6.2 Human reservoirs

As humans only excrete *Campylobacter* during an acute infection and up to 3 weeks after recovery most human *Campylobacter* infections are classified as single, sporadic cases or as part of small family related outbreaks (Harris *et al.*, 1986; Skirrow, 1991; Anderson *et al.*, 2003). Therefore human carriers are considered only a minor reservoir for *Campylobacter* infections and human-to-human transmission is considered infrequent in developed countries. On the contrary, Blaser *et al.* (1983) report that secondary transmission to other members of a household with an infected person has been accounted for at different rates, ranging from 0-20% of infections.

Campylobacteriosis has been found in homosexual men with certain sexual practices being identified as a route of transmission. Neonates might become infected during or shortly after delivery because of the faecal contamination of the birth canal, even in cases where the mother did not have a recent history of diarrhoea (Blaser *et al.*, 1983). *Campylobacter* bacteraemia during pregnancy may lead to intrauterine infection of the foetus with subsequent abortion, stillbirth or early neonatal death (Smith, 2002).

However, in developing countries, it is suspected that carriage by humans plays a larger role in the transmission of infection than in developed countries (Blaser *et al.*, 1983; Sinell, 1985; Reintjes *et al.*, 1999; Anderson *et al.*, 2003). Atanassova and Ring (2000a) report a carrier rate of *Campylobacter* in humans of 30% in developing countries compared to 1% in developed countries.

6.3 Inanimate reservoirs

Surface water can become contaminated with *Campylobacter* through faeces of wild or domesticated birds and animals (Blaser *et al.*, 1983). Due to the optimum environmental conditions for the pathogen there, *Campylobacter* spp. can survive for a long time in contaminated water (Atanassova and Ring, 2000a). Consumption of this water untreated as drinking water by humans is a serious health hazard and has led to large outbreaks of *Campylobacter*-enteritis in the past (Doyle, 1981; Griffiths and Park, 1990; Skirrow, 1991; Simango *et al.*, 1992; Diergaardt, 2001;).

Mud and sewage sludge has been tested positive for *Campylobacter*, indicating that the organism can survive in faeces and contaminated soil when environmental conditions are right and thus be a source of infection for humans (Blaser *et al.*, 1983; Atanassova and Ring, 2000a).

6.4 Transmission to humans

6.4.1 Direct transmission

Persons whose occupation brings them into close and regular contact with animals and animal products seem to be at increased risk of infection. These include farmers, veterinarians, laboratory technicians, abattoir workers, poultry processors and butchers (Blaser *et al.*, 1979; Harris *et al.*, 1986; Skirrow, 1991; Rosenquist *et al.*, 2003). It appears that many of the professionals exposed to the pathogen develop a solid immunity (Bryan and Doyle, 1995). A study performed by Jones and Robinson revealed a positive *C. jejuni* titer in 27-68% of workers with contact to poultry and cattle but only in 2-5% of persons not exposed (1981, cited by Blaser *et al.*, 1983). A study performed among Swedish poultry abattoir workers revealed that permanent staff acquires immunity to *C.*

jejuni compared to part-time staff members that do not show immunity (Bryan and Doyle, 1995).

Pet owners may contract the disease by contact with infected dogs or cats with puppies and kittens bearing the higher risk of contamination and infection of owners, especially for children (Blaser *et al.*, 1983; Griffiths and Park, 1990; Doyle and Roman, 1991; Skirrow, 1991). These routes of infection are well known and documented but they are of minor importance in the transmission of *Campylobacter* enteritis to humans (Skirrow, 1991, Thurm and Dinger, 1998).

6.4.2 Indirect transmission

Campylobacter enteritis is a typical food-borne zoonosis. *Campylobacter* are introduced either via meat or milk. While milk is normally responsible for larger group outbreaks, meat is considered to be responsible for sporadic infections (Skirrow, 1990; Reintjes *et al.*, 1999).

Although red meat and offal are considered a possible cause for human infection, poultry meat is the product with the highest contamination rate and therefore is seen as the major vehicle of *Campylobacter* to humans (Skirrow, 1991; Atanassova and Ring, 2000a; Rosenquist *et al.*, 2003). Occasionally other food is mentioned as being of concern including vegetables, fruits, raw fish or shellfish and fresh mushrooms (Blaser *et al.*, 1979; Doyle, 1994, BgVV, 1998). Even recreational activities in the environment are described as a risk factor for humans to contract campylobacteriosis (Wong *et al.*, 2004). It is however generally accepted that the contamination of raw poultry meat and the subsequent cross-contamination of ready-to-eat food in the consumers' homes or in public preparation bears the highest risk of infection to humans.

Milk: Cows' milk was the cause in most milk-related outbreaks of campylobacteriosis, but goats' milk has also been implicated. Surveys have shown that 4.5-5.9% of cows' milk samples may be contaminated with *C. jejuni* (Skirrow, 1991). *Campylobacter* get into milk usually by faecal contamination during the milking process while bovine *Campylobacter* mastitis is a rather uncommon cause of human campylobacteriosis (Skirrow, 1990; Atanassova and Ring, 2000a). The distribution of raw or insufficiently pasteurised milk has reportedly led to large outbreaks in the past (Skirrow, 1990; Skirrow 1991; BgVV, 1998; Rosenquist *et al.*, 2003).

Poultry meat: Poultry meat is a well-established reservoir of *Campylobacter jejuni/coli* and contamination with 10^2 to 10^4 CFU/g (colony forming units), (Doyle, 1994) is common. According to Skirrow (1991) the counts of bacteria on broiler carcasses can

even reach up to 2.4×10^7 CFU/g depending on the kind of carcass processing. The main predilection sites on chicken carcasses are the skin of the neck, breast and thighs. No *Campylobacter* was detected in muscle tissue (Berndtson *et al.*, 1992; Kotula and Pandya, 1995; Berrang and Buhr, 2001). Data obtained from countries where the incidence of human campylobacteriosis has declined support the fact that the consumption of chicken meat and products are an important source of infection. In Belgium, for example, there was a decrease in the incidence of human campylobacteriosis along with the dioxin crisis in June 1999, probably because chicken and other meat products were withdrawn from the shops (Rosenquist *et al.*, 2003).

Although most authors state that *Campylobacter* do not multiply in food, Lee *et al.* (1998) found that *C. jejuni* replicate quickly on chicken skin stored at 4°C and ambient room temperature, resulting in high numbers of organisms present after only a few days of storage. Moreover, Lee's findings suggested that *C. jejuni* can survive freezing and thawing, and that the contamination can exceed the infective dose when food products are left in the thawed state at refrigeration temperatures for long periods.

As the infection dose of *Campylobacter* is very low with only 400 to 500 bacteria, the initial contamination of a product is generally high enough to cause campylobacteriosis in humans (Shanker *et al.*, 1982; Griffiths and Park, 1990; Bryan and Doyle, 1995). Therefore, ingestion of even lightly contaminated food can already cause infection in humans.

The three common routes of transmission via contaminated poultry meat are: Handling of raw meat, consumption of raw or undercooked meat and products, and cross-contamination of other, ready-to-eat foods like bread or salad (Sinell, 1985; Skirrow, 1990; Thurm and Dinger, 1998). These three ways of contamination are present in commercial kitchens as well as in the consumers' households and cannot readily be separated from each other in terms of the risk they pose to the consumer (Anderson *et al.*, 2003). The pathogens enter the kitchens on frozen and chilled raw poultry and in the associated thaw and drip water. Wong *et al.* (2004) isolated *Campylobacter* from the outside of 24% of chicken packs, which indicates that even the packs could be an important source of cross-contamination and infection for humans, for instance packers in retail facilities, consumers and so forth.

Human infections are often associated with a lack of kitchen hygiene. Improper cleaning of hands, working surfaces or kitchen utensils like cutting boards and knives after contact with raw poultry increases the risk of cross-contamination (Harris *et al.*, 1986). Cloths and sponges become contaminated when they are used to wipe up drip and thaw fluid and

smears from poultry parts or carcasses, working surfaces or kitchen utensils. On these cloths, the microorganisms may multiply under favourable environmental conditions and might be spread further to working surfaces, kitchen utensils and hands of the users (Bryan and Doyle, 1995). Anderson *et al.* (2003) regard the drip fluid as one of the major hazards for cross-contamination of *Campylobacter* in the kitchen and base one of their risk assessment models on it (Figure 1.2).

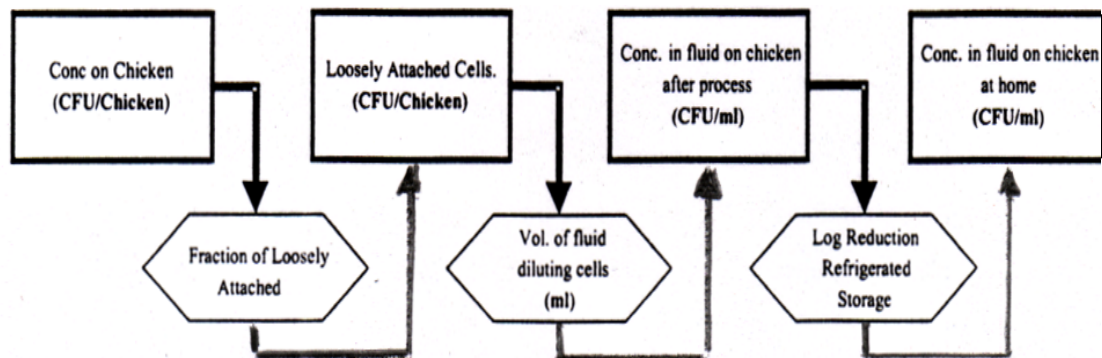


Figure 1.2 Drip concentration calculation schematic for the risk assessment of *Campylobacter* cross contamination

7 Seasonal trends

Research findings regarding the prevalence of *Campylobacter* in broilers and in fresh poultry meat, range from 0% to 100%. One of the causes for this wide difference in the prevalence is seen in the seasonal variations in contamination rates (Stern, 1995; Willis and Murray, 1997; Atanassova and Ring, 1999).

Several authors described a seasonal pattern in reported cases of poultry infections as well as cases of human campylobacteriosis with a definite peak in warmer months. In principle, the highest contamination of poultry flocks and poultry meat is reported in summer and early autumn while contamination rates are low in winter and early spring (Blaser *et al.*, 1979; Blaser *et al.*, 1983; Skirrow, 1991; Berndtson *et al.*, 1996b; Atanassova and Ring, 1999). Jacob-Reitsma *et al.* (1994) described a relationship between elevated temperatures and high *Campylobacter* isolation rates in poultry flocks. According to these authors, the seasonal variation in the contamination of broiler flocks with *Campylobacter* might be one of the explanations for the summertime peak found in human campylobacteriosis. In Sweden the summer peaks were corresponding to the return of travellers from abroad (Blaser *et al.*, 1983; Skirrow, 1991).

Atanassova and Ring (1999) found no seasonal variations in broiler flocks while kept under constant environmental conditions (i.e. temperature and humidity) compared to isolation peaks in summer and low contamination rates during winter in production units without standardised climatic conditions. Willis and Murray (1997) saw a definite correlation between seasonal variations in the contamination rates of broiler flocks and the same seasonal pattern in contaminated poultry carcasses and products.

In the former Zaire, now Democratic Republic of Congo, where mean temperatures are constant throughout the year, isolation of *Campylobacter* from patients with diarrhoea was much more frequent in the wet than in the dry season. However, early studies in South Africa have shown a summertime peak in *Campylobacter* infections (Blaser *et al.*, 1983). In a survey conducted at the Red Cross Children's Hospital in Cape Town in the 1980's, potential diarrhoea causing agents were found in 40% of summer cases and in 70% of winter cases. The organisms isolated were *Campylobacter*, enteropathogenic *E. coli*, *Shigella*, *Salmonella*, rotavirus, adenovirus and *Cryptosporidium*. No detailed description of the percentage of each of the organisms within the isolates or an explanation regarding the variation in isolation of pathogens is given in this study (Ireland, 1998).

8 Methods to detect *Campylobacter* spp. in food

Conventional detection of *Campylobacter* in food depends on selective cultural enrichment followed by isolation from selective agar. Identification and confirmation is based on biochemical tests. These methods are time consuming and laborious and require an average time of 4 – 6 days. This time range is considered as too long especially for the detection of causes of suspected food borne illnesses.

DNA hybridization and polymerase chain reaction (PCR) have been developed as a rapid, sensitive and reliable alternative to detect *Campylobacter* in food samples. This method allows first results within 48 hours. Several PCR assays, with and without pre-enrichment, have been described in literature (Giesendorf *et al.*, 1992; Hazeleger *et al.*, 1994; Winters and Slavik, 1995; Docherty *et al.*, 1996; Ng *et al.*, 1997; Waage *et al.*, 1999, Thunberg *et al.*, 2000).

Ring and Atassanova (1999) performed a comparison study of conventional methods and PCR based assays to detect *Campylobacter*, which revealed the higher sensitivity and detection rate of the latter method.

Most studies described in the literature review are based on the use of washes or swabs taken from chicken samples (Smeltzer, 1981, Furlanetto *et al.*, 1991; Stern and Line, 1992; Winters and Slavik, 1995, Ng *et al.*, 1997; Shih, 2000). The findings of Thomas and McMeekin (1980) revealed that rinses or washes and even swabs might not remove all contaminating bacteria because they are partly trapped in feather follicles, channels and folds of skin of chicken carcasses or products. Based on these results the use of homogenized sample pieces is expected to be more effective to detect *Campylobacter* in chicken samples.

8.1 Culture based isolation methods

Campylobacter are thermophilic, slow growing and micro-aerobic, therefore samples have to be incubated at 5-7% O₂, 10% CO₂ and 85% N₂ for 48 hours. Either direct plating or enrichment plating using selective or non-selective media can be used to culture food samples. While most studies showed no significant difference in results obtained from selective direct or enrichment plating some authors found that enrichment will increase the recovery of organisms in samples with only slight contamination (Furlanetto *et al.*, 1991; Stern and Line, 1992; Aquino *et al.*, 1996; Hunt *et al.*, 1998; Shih, 2000; Line *et al.*, 2001).

Selective media are supplemented with combinations of different antibiotics to suppress the growth of contaminating microorganisms in the samples. A sensitive factor in pre-enrichment is the time used. If the enrichment period is too short the level of microorganisms might still be too low for detection, while a period too long can result in an overgrowth of contaminating bacteria. Different types of media can be used, some of which are listed below:

8.1.1 Selective Agar

1. Modified charcoal cefoperazone deoxycholate agar (mCCDA), contains cefoperazone and amphotericin as selective antibiotics
2. Mueller – Hinton blood agar (MHBA)
3. Blaser's selective agar
4. Skirrow's selective agar, contains vancomycin, polymyxin B and trimethoprim as selective antibiotics
5. Campy-Cefex agar (CCA), contains cefoperazone and cyclohexamide

8.1.2 Selective Enrichment Broth

1. Bolton broth + selective antibiotics
2. Hunt broth + selective antibiotics
3. Preston broth + selective antibiotics
4. Rosef's broth + selective antibiotics

8.2 Molecular method: Polymerase Chain Reaction (PCR)

The PCR was developed in 1983 and since then has become a standard molecular biological method for diagnostic and research (Steffan and Atlas, 1991). Recovery of bacteria from foods based on PCR techniques permits a more rapid and sensitive detection and identification of *Campylobacter* without the need for conventional culturing (Hill and Jinnemann, 2000).

8.2.1 Principles of PCR

While conventional biochemical and immunological methods make use of the presence of gene products like antigens or metabolic end products in the tested material, PCR identifies microorganisms based on their specific gene structure.

PCR analysis uses the specific physical and morphological characteristics of the double helix conformation of DNA molecules. The three basic steps of a PCR – denaturation, annealing, and extension – can be repeated many times using the new DNA sequences as templates for the next cycle and results in an exponential increase of the target DNA, known as amplification. Even very low concentrations of specific sequences of DNA in heterologous mixtures of genetic material can therefore be detected and identified (Steffan and Atlas, 1991).

To increase the specificity and efficiency of the process, a second, subsequent PCR analysis can be performed based on the PCR products obtained from the first one. This PCR can be performed as a nested or as a semi-nested PCR (Waage *et al.*, 1999; Hill and Jinnemann, 2000; Theron *et al.*, 2001).

8.2.2 The use of PCR to detect *Campylobacter* spp. in poultry

The use of a PCR analysis for the detection of bacterial DNA in food samples has been proven to be more problematic than for DNA isolation from clinical samples. Various different sources of DNA usually exist in food samples with high levels of background flora, and the level of target DNA might be very low compared to other DNA present. Furthermore, the sample might contain substances that are inhibitory to the PCR process. Thus, the DNA must be isolated and purified before the PCR analysis (Waage *et al.*, 1999; Hill and Jinnemann; 2000).

In food samples, enrichment prior to PCR is often used although it prolongs the time of the analysis. Several authors described the enrichment of poultry samples prior to bacterial lysis and DNA extraction as a necessary step to increase the number of viable and cultivable target organisms. It is also claimed that enrichment dilutes PCR inhibitors and dead or non-culturable cells (Giesendorf *et al.* 1992; Stern and Line, 1992; Docherty

et al., 1996, Waage *et al.*, 1999; Denis *et al.*, 2001). The enrichment time varies according to the sample and broth used between 18 and 48 hrs (Giesendorf *et al.*, 1992; Stern and Line, 1992; Ng *et al.*, 1997, Waage *et al.* 1999).

Mandrell and Wachtel (1999) describe several nested or multiplex PCR assays without a prior enrichment step. These assays could detect as low as 35 to 120 cells per ml in a completion time of 24 hours.

Furlanetto *et al.* (1991), however, claimed that they did not find a significant difference between enrichment procedures and direct selective plating and subsequently concluded that there is no need for using enrichment broth for recovering *C. jejuni* from chicken carcasses.

Table 1. 2 A summary of published data of the prevalence of *Campylobacter* contamination of poultry products

Samples	Sample size	Prevalence	Detection method	Author, year	Season
Breast and thighs, chicken *	64	47.5% (a)	BC (with enrichment) (a)	Aquino <i>et al.</i> , 1996	n.k.
		95% (b)	BC (without enrichment) (b)		
Skin, livers, neck, broilers*	111	45.9%	BC (with enrichment)	Atanassova and Ring, 1999	3 years
Neck skin *	100	89%	BC (with enrichment)	Berndtson <i>et al.</i> , 1992	n.k.
Peritoneal cavity swabs *	100	93%			
Subcutaneous samples (feather follicles) *	100	75%			
Neck skins and organs *	49	56%	PCR (with enrichment)	Denis <i>et al.</i> , 2001	Autumn
Various portions and organs **	70	17.5%			Spring
Carcass washes, chicken**	42	38%	BC (with enrichment)	Furlanetto <i>et al.</i> , 1991	n.k.

Samples	Sample size	Prevalence	Detection method	Author, year	Season
Skin samples, chicken *	45	80%	PCR (with enrichment)	Giesendorf <i>et al.</i> , 1992	n.k.
Carcass rinses, chicken *	50	52%	BC (with enrichment)	Jones <i>et al.</i> , 1991	n.k.
Carcass rinses **	98	31.6%			
Carcass rinses *	44	50% (<i>C.jejuni</i>) 23% (<i>C.coli</i>)	BC (without enrichment)	Joseph <i>et al.</i> , 1989	n.k.
Swabs from chicken carcasses *	80 (4x20)	20: 85% 20: 85 20: 80% 20: 70%	BC (without enrichment)	Juven and Rogol, 1986	n.k.
Skin of breast, thighs and drumsticks, chicken *	40	62.5%, 45% and 50%, respectively	BC (without enrichment)	Kotula and Pandya, 1995	n.k.
Carcass swabs (poultry) *	839	Chicken 80% Ducks 48% Geese 38% Turkeys 3%	BC (without enrichment)	Kwiatek <i>et al.</i> , 1990	n.k.
Carcass rinses and neck skin, chicken	739	71%	BC (with enrichment)	Meldrum <i>et al.</i> , 2004	1 year, peak in summer (June) and lowest rates in winter/spring (January, March, December)
Chicken rinses **	4	0%	PCR (with enrichment)	Ng <i>et al.</i> , 1997	n.k.
Chicken carcasses *	120	49%	BC (with enrichment)	Oosterom <i>et al.</i> , 1983	n.k.
Livers *	40	73%			
Stomachs*	20	50%			
Hearts*	20	65%			
Chicken rinses*	50 (a) 50 (b)	62% (a) 54% (b)	BC (with enrichment)	Park <i>et al.</i> , 1981	n.k.

Samples	Sample size	Prevalence	Detection method	Author, year	Season
Chicken carcasses, portions, livers and gizzards **	95	71%	BC (with enrichment)	Shih, 2000	n.k.
Carcass rinses *	50	94% (a) 84% (b)	BC (with enrichment)(a) BC (without enrichment) (b)	Smeltzer, 1981	n.k.
Carcass rinses *	40	45%	BC (without enrichment)	Shanker <i>et al.</i> , 1982	n.k.
Poultry carcasses and products**	733	25.6% (carcasses) 40% (products)	n.k.	Uyttendaele <i>et al.</i> , 1999	14 months
Rinses of chicken packs (outsides)*	300	24%	PCR (with enrichment)	Wong <i>et al.</i> , 2004	n.k.
Chicken carcass rinses*	360	69%	BC (with enrichment)	Willis and Murray, 1997	12 months, peak in June/July (summer) and lowest in winter (December)

* Samples taken at abattoir

** Samples taken at retail outlets

BC: bacteriological culture

PCR: polymerase chain reaction

n.k.: not known

CHAPTER 2

Processing of poultry and dissemination of *Campylobacter*

1 Introduction

Poultry slaughtering differs from the slaughter process used for red meat animals, resulting in unique microbiological consequences. Industrial, large-scale poultry slaughter and processing is a multi stage operation and the basic process is virtually the same worldwide. The major emphasis is on speedy and cost effective production with prevention of cross-contamination being of less importance (Humphrey, 1991).

Modern slaughter lines can operate at processing speeds of 6000 carcasses or more per hour on a single line. With high-rate processing, the carcasses on the line are very close together and cross-contamination occurs readily (Mead, 2000; Alter *et al.*, 2005). Cross-contamination can occur during the transport from the farm to the processing plant and at many points on the slaughter line. Different populations of the pathogen may be carried into the processing plant by successive broiler flocks, and the same strains of *Campylobacter* may be recovered from different poultry processing operations. However, *Campylobacter* seems to be unable to colonise equipment in the processing facility and contaminate broilers from flocks processed at later dates in the plant (Hinton *et al.*, 2004). Certain stages during the processing, however, are of particular importance (Humphrey, 1991).

The following is a résumé of the main stages of the poultry slaughter process as described in the literature. It has, however, to be stated that the processing can differ between plants in various ways.

2 Outline of poultry processing

Birds are transported to the abattoir in special containers or crates. After arrival at the abattoir, the birds are taken out of the transport crates and hung manually by the legs onto a continuously moving system of shackles. They are stunned by a low voltage electrical shock in a water bath. Electrical stunning of the birds is effected as their heads touch a brine solution to complete an electric circuit, causing unconsciousness with or without cardiac arrest at the same time (Kallweit *et al.*, 1988).

Next, they proceed to a neck cutting and bleeding stage. The neck is partially cut either by hand or automatically with a rotating knife-blade. In case of the use of a mechanical throat-cutting device a worker is required to hand-cut any bird the machine has missed (Silverside and Jones, 1992).

After bleeding the birds are immersed in hot water to facilitate subsequent plucking. Two different scalding regimens are used depending on the type of product, which is either chilled or frozen (Kallweit *et al.*, 1988):

Soft or mild scalding is required for birds that are sold as chilled fresh products. The low water temperature used (49° - 52°C) only softens the skin and avoids damages during subsequent defeathering processes (Humphrey, 1991).

Hard scalding is used on birds being sold frozen. At a water temperature of 58° - 60°C the carcass skin is softened and partly loosened. Consequently, during plucking the epidermal layer is removed producing a white-skin-carcass (Humphrey, 1991).

After scalding the carcasses are defeathered by passing several on-line plucking machines. These consist of drums with flexible rubber fingers in different sizes and settings for rough- to fine-plucking. Feathers are removed by a scouring action (Mead, 2000). Some processing lines include a singeing stage to remove hair-like fine feathers and appendages. There, each carcass passes through a sheet of flames as it moves along the conveyor line (Mountney and Parkhurst, 1995).

After washing and removal of head and feet by means of automatic head and foot cutters, the carcasses pass along a chute and through rubber curtains from the dirty side of the plant to the evisceration line, the clean part. Evisceration is mechanized with different machines involved. In principle, guts are removed in two steps. First the intestines are sucked out of the carcass through a circular incision around the vent by vacuum. Secondly, the viscera are lifted and removed by a fork-like device. Afterwards the lungs are removed by vacuum. During and after evisceration the carcasses are spray - washed to remove any spoilage with blood and faeces (Mead, 2000).

Next the birds are chilled, either by water immersion or air blasting. **Water immersion chilling** is a continuous in-line process and carcasses move through one or more large tanks of water, to which ice or chilled water is added. Air is sometimes introduced at the bottom of the tanks to improve agitation that facilitates the cooling and removes some of the contaminating microorganisms. The water in the tanks can flow with the direction of carcasses (through-flow system) or the birds are moved mechanically against the flow of incoming water (counter-flow system). The latter one has the advantage that the

carcasses meet the cleanest water when they leave the system minimizing cross-contamination and decreasing bacterial counts on carcasses. Birds have to be re-hung manually when they leave the chilling tank, and an adequate drip-time afterwards is essential. This system is very efficient for rapid chilling of small carcasses and is mainly used for hard-scalded birds that are sold as frozen products (Richardson, 1991; Allen *et al.*, 2000).

Alternatively, birds are soft-scalded and air-chilled before sold as fresh. **Air chilling** is basically a dry process, utilizing cold air, either in a chill-room (batch process) or by continuously moving carcasses through an air-blast tunnel. A modified air chilling system incorporates fine water sprays in the first stage of cooling (evaporative air chilling). With this method, the extra cooling effect of water evaporating from the carcass surface is utilized and carcass weight loss and surface dehydration are minimized. Air chilling can be used for small carcasses as well as for large birds such as turkeys (Richardson, 1991; Allen *et al.*, 2000).

After chilling the carcasses are re-weighed, graded and packed or transferred for further processing prior to chilling or freezing (Kallweit *et al.*, 1988).

Figure 2.1 gives a general illustration of the flow of products during poultry processing. In South Africa, however, feet, heads and intestines (rough offal) are edible products of the poultry processing as well as necks, livers and hearts (red offal).

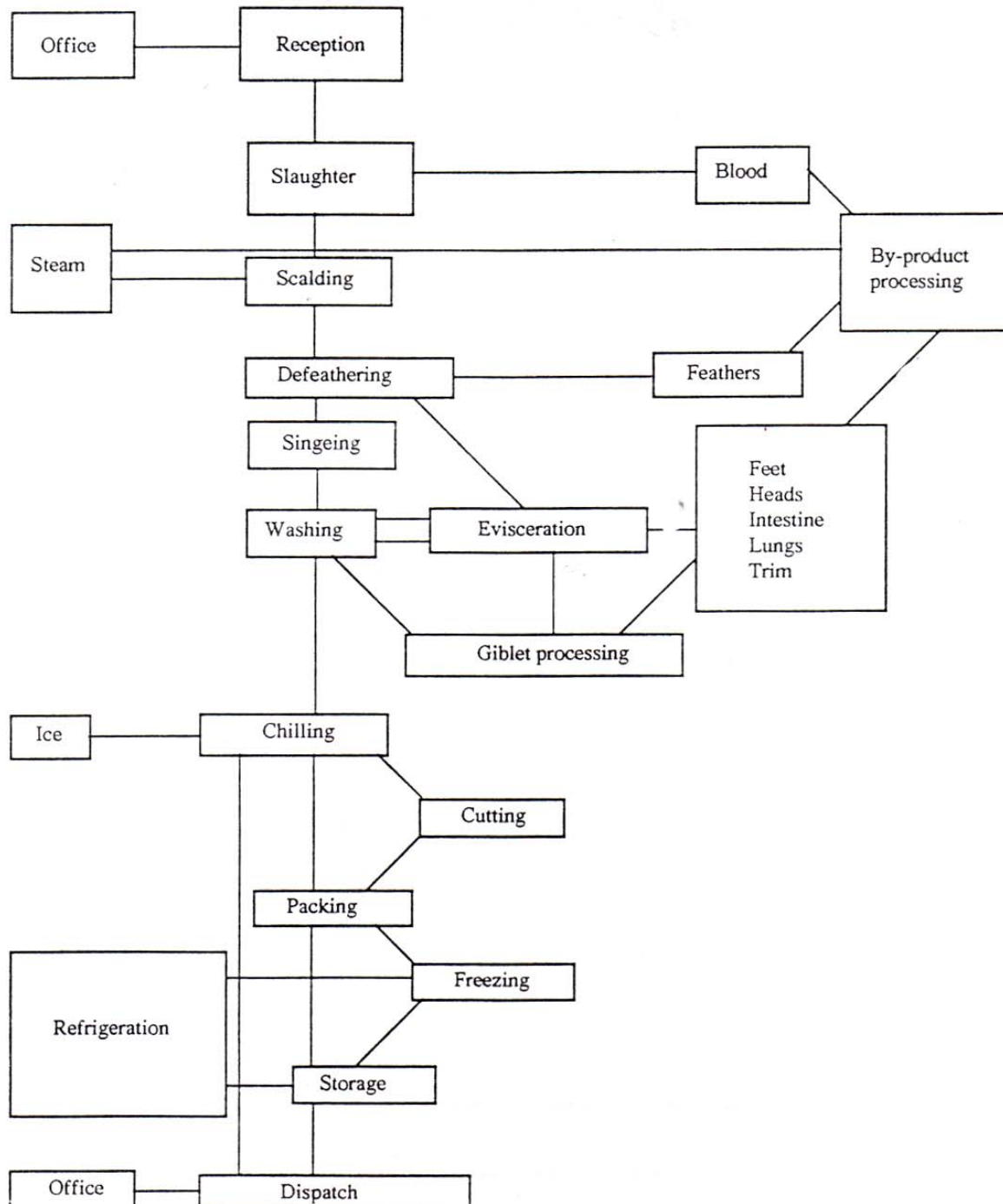


Figure 2.1 Flow diagram of poultry processing (Silverside and Jones, 1992)

3 Dissemination of *Campylobacter* during processing

Due to the high infection rate with *Campylobacter* in broilers, even healthy chickens are asymptomatic carriers of a huge amount of the bacteria. When birds arrive at the processing plant, they carry a large microbial load, and the organisms are present in the intestine but also on feet, feathers and skin of the living bird. The process of converting a live bird into an oven-ready product leads to the removal of a large proportion of the microorganisms but further contamination of carcasses, cross-contamination and multiplication of *Campylobacter* can occur at any processing stage (Kotyla and Pandya, 1995; Mead, 2000).

During processing, the carcasses pass through a series of operations where contamination can occur from the equipment of the plant, hands of workers and cross-contamination from other birds (Humphrey, 1991; Bryan and Doyle, 1995).

Also, the skin is not removed during processing and is already heavily contaminated in the living bird. Due to the anatomic features of the bird's skin the microorganisms are not removed during processing but entrapped in the follicles, folds and channels, thus forming a permanent source of contamination during the process (Thomas and McMeekin, 1980, Izat *et al.*, 1988; Mead, 1991b; Mead *et al.*, 1994; Geornaras *et al.*, 1994; Saleha. *et al.*, 1997; Mead, 2000). Bacteria also adhere to the skin surface and will subsequently form a biofilm that is difficult to remove. The organisms are largely protected from biocidal activities during the slaughter and processing within that biofilm (Alter *et al.*, 2005).

The degree of contamination of carcasses varies considerably at different stages of processing. The stages that most influence the *Campylobacter* status of the product at the end of processing are transport, scalding, plucking, evisceration and chilling (Thomas and McMeekin, 1980; Anderson *et al.*, 2003).

Transport crates are often contaminated with *Campylobacter* even after cleaning and disinfection, resulting in contamination of the next load of broilers (Jacob-Reitsma, 1997). Furthermore, transport-induced stress increases the shedding of *Campylobacter spp.* in faecal material of broilers that may subsequently result in extensive carcass contamination (Whyte *et al.*, 2001).

During scalding, loose microorganisms are washed from feathers, feet and skin into the scalding water. Depending on the water temperature they either get killed or survive and redistribute on the same or other carcasses. Organisms attached to the chicken skin are

only insufficiently removed or killed during scalding. After scalding the skin surface retains a film of water that includes soluble organic matter and large populations of microorganisms. While the overall load of *Campylobacter* on the single carcass is reduced, cross-contamination in the scalding tank is considered as a critical point in processing (Genigeorgis *et al.*, 1986; Izat *et al.*, 1988; Humphrey, 1991; Jones *et al.*, 1991; Anderson *et al.*, 2003).

The mechanised defeathering process damages the surface skin to a certain extent depending on the scalding temperature and the time of immersion. Considerable contamination occurs either from carcass to carcass or is transferred by the plucking equipment. The beating of the rubber fingers on skin surfaces pushes pathogens into skin follicles and folds where they get trapped and cannot easily be removed by following washing procedures (Thomas and McMeekin, 1980; Mead, 2000; Anderson *et al.*, 2003).

Evisceration is even more important with respect to cross-contamination of carcasses and contamination of equipment than scalding and defeathering, resulting in an increase of *Campylobacter* on the carcasses (Genigeorgis *et al.*, 1986; Jones *et al.*, 1991; Mead *et al.*, 1994; Bryan and Doyle, 1995).

The two chilling methods used have different advantages and disadvantages. The number of bacteria present in the chilling water is positively related to the number of organisms present on the carcass skin. The advantage of water immersion spin chilling is definitely the washing effect on the carcasses, resulting in a reduction of *Campylobacter* on the carcasses' surface. The disadvantage is the build up of bacteria in the water, which can lead to recontamination if no adequate measures for water disinfection are implemented. Secondly, water chilling by immersion of carcasses leads to significant changes in the micro-topography of the skin. Skin swelling associated with the uptake of water by skin tissue can trap bacteria already located in deep channels and crevices and render them less accessible to physical and chemical removal. Alternatively, channels and folds are opened and exposed to contaminants present in the chilling water, increasing the level of contamination. In addition the water uptake causes a high water activity (a_w), which results in a short shelf life even with proper refrigeration (Kallweit *et al.*, 1988; Richardson, 1991; Kraemer, 1992; Allen *et al.*, 2000).

Dry air chilling in principle results in a reduction of bacteria on the carcass surface and body cavity. The water activity initially is lower as an effect of the drying out of the surface during blowing. But moisture migration from deeper tissues onto the surface during storage results in the same shelf life as that for water chilled poultry. Air chilling using evaporation causes pools of water remaining in the body cavities presenting an

ideal moist environment for *Campylobacter*. In general the contamination of air-chilled poultry is higher than that of properly water chilled birds because cross-contamination occurs via physical contact of carcasses and microorganisms circulating in the cold air (Thomas and McMeekin, 1980, Kallweit *et al.*, 1988; Richardson, 1991; Kraemer 1992; Silverside and Jones, 1992; Stern, 1995; Allen *et al.*, 2000; Mead, 2000).

4 Reduction of *Campylobacter* contamination on broiler carcasses

The elimination of *Campylobacter* from poultry requires control measures at all stages of the food chain, from agricultural production on the farm, to processing, manufacturing and preparation of foods in commercial establishments and the domestic environment, the households. Specific intervention methods on the farm are aimed at the reduction of *Campylobacter* incidence in poultry to avoid horizontal transmission of the pathogen from the environment to the flock of birds. However, intervention measures prior to processing have so far proven to be of limited effect. Therefore, decontamination procedures within facilities will remain the primary line of defence in eradicating *Campylobacter* from poultry products (WHO, 2000; Kemp *et al.*, 2002).

Especially the plucking (defeathering) and evisceration as well as the chilling process are the stages during processing with the highest risk of cross contamination thus having a direct impact on the safety and quality of the final product (Li *et al.*, 1995).

Chlorination of water supplies in poultry processing has been used for many years as an aid to reduce the contamination of carcasses and cross-contamination (Mead *et al.*, 1975). Chlorine is used in different concentrations as addition to poultry chiller water and spray water used during processing to control microbial populations in the chiller water and to improve the shelf life of the final product. Chlorination has been the method of choice because of its efficacy, availability and relatively low cost (Tsai *et al.*, 1992).

The concentration of free chlorine or total residual chlorine in the chiller water determines the rate of reduction of pathogens in poultry meat. Chlorine reacts with microorganisms in the water, but also with inorganic and organic materials present. After chlorine is added to the chiller water, the properties of the water like pH, temperature and solids and dissolved compounds determine the amount of chlorine that is consumed during a certain time period, known as chlorine demand. Equilibrium must be kept in the chiller water to maintain a constant level of 20 to 50 ppm chlorine for an efficient disinfecting action of chlorine in the water (Bailey *et al.*, 1986; James *et al.*, 1992, Tsai *et al.*, 1992, Allen *et al.*, 2000).

Other methods, like the addition of other chemicals such as trisodium phosphate, organic acids and ozone to operational water for the microbiological control in poultry processing have been studied. However, these various methods have been regarded as unacceptable for industrial use due to various reasons (Li *et al.*, 1995).

Contrary to that, Kemp and Schneider (2002) describe the pre-chill effect of Acidified Sodium Chlorite (ASC) on the reduction of *Campylobacter* on broiler carcasses. ASC is a FDA/USDA approved disinfectant that in combination with carcass washing proves to be effective in the control of *Campylobacter* on broiler carcasses.

The use of ultrasonics combined with heat treatment for the decontamination of poultry has been described although the feasibility for industrial use remains questionable (Lillard, 1994).

Berrang *et al.* (2000) describe the effect of an additional, second scalding step after defeathering on microbial levels on carcasses. However, a second scalding treatment gentle enough not to change the carcass characteristics or the meat quality would not effectively lower the rate of *Campylobacter* on poultry.

In order to prevent and minimise food safety hazards, the importance of the *hazard analysis critical control points* (HACCP) concept cannot be overemphasised. HACCP is a simple but highly specialised method for the identification and control of potential hazards with the aim to prevent food safety hazards from occurring and to improve product quality and shelf life. In food production, HACCP focuses on preventing potential food safety hazards rather than detecting problems in the final product. It relies on science to identify and prioritise potential food safety risks such as microbiological, chemical and physical contamination (Tompkin, 1990; Mountney and Parkhurst, 1995).

CHAPTER 3

Materials and methods

1 Pilot study

A pilot study was first performed to determine the extraction method, the specificity and sensitivity of the different primers, the annealing temperatures and number of cycles used in both, the first and the nested, PCR steps. No differentiation between the detection of *C. jejuni* and *C. coli* by the PCR used was made in this study. As the majority of gastroenteritis infections in humans is caused by *C. jejuni*, a culture of *C. jejuni* obtained from a dog was used in the pilot study as well as the positive control in the field study (Petersen and Newell, 2001; Rosenquist *et al.*, 2003).

1.1 Cultivation and quantification of bacteria

A culture of *C. jejuni* obtained from a dog was used to determine the specificity and sensitivity of the primers in experimentally infected chicken material (skin and organs).

The *Campylobacter jejuni* strain used was cultivated anaerobically at 42°C on non-selective (blood) agar. Subsequently, the bacteria were diluted in sterile phosphate-buffered saline (PBS) to a concentration of approximately 10^7 cells per ml. The optical density of the solution was determined to be about 0.226 at a wavelength of 535 nm by using a LKB Biochrome Ultrospec II spectrophotometer.

Of this undiluted (pure) culture solution, 10-fold dilutions in PBS up to 10^{-6} were done. To determine the quantity of cells in the dilutions, a direct cell count using Breed's direct smear method in a 100 mm² chamber was then performed as follows:

For counting, 10 µl of the 10^{-1} dilution was used. This was transferred onto the Breed's chamber, consisting of a 100 mm² field with subfields of 0.25 mm² each. The cells in 10 subfields were counted and the average amount of cells for the subfields was determined (N). The amount of cells per ml of undiluted bacterial solution was calculated by using the following formula:

$N \times 4 \times 10^4$ cells per ml

N = 146 cells per subfield (average)

(4) = fold of 0.25 mm² (total of 100 mm²)

(10⁴) = 1 mm² → 100 mm² (correlates to 0.01 ml → 1 ml)

According to the calculation, the undiluted solution of *Campylobacter jejuni* contained 5.84×10^7 cells/ml. Subsequently, the concentrations of the bacterial dilutions ranging from 10^{-1} to 10^{-6} were determined as shown in Table 3.1.

Table 3.1 Quantification of bacterial dilutions for PCR sensitivity and specificity tests

	Not diluted	Dilutions					
Concentration of solutions	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Cells per ml	$5,84 \times 10^7$	$5,84 \times 10^6$	$5,84 \times 10^5$	$5,84 \times 10^4$	$5,84 \times 10^3$	$5,84 \times 10^2$	$5,84 \times 10^1$

A 200 µl volume of each solution was used for direct bacterial DNA extraction as discussed below and a further 200 µl volume of each dilution was used to spike poultry samples. Each bacterial dilution and each spiked sample was subsequently examined by PCR.

1.2 Extraction method

The QIAamp DNA Mini Kit (QUIAGEN GmbH, Hilden, Germany) was used for DNA extraction of bacteria and poultry samples, and the method referred to as Tissue Protocol in the Kit Manual (02/2003) was followed with slight modifications. As we dealt with solid tissue samples, the incubation period of the samples with Proteinase K (QUIAGEN GmbH, Hilden, Germany, part of the DNA Mini Kit) at 56°C had to be prolonged. All samples and all bacterial dilutions were thus incubated overnight for approximately 18 hours to achieve complete lysis.

1.3 Preparation of tissue samples

In this study, liver tissue, intestines and skin of poultry were used. Each skin sample consisted of a pool of 5 samples from different sites on the carcass, i.e. neck, both thighs and both sides of the breast. Of each sample, 25 mg was weighed into a petridish and cut up into very small pieces before the extraction was performed. To minimise the risk of cross contamination, the whole process was performed aseptically by using sterile equipment for each sample.

1.4 Spiking of poultry samples

For the spiking of samples and for use as negative tissue controls with the PCR, a specific pathogen free (SPF) chicken was obtained from the Department of Poultry

Diseases, Veterinary Faculty, University of Pretoria. However, the bird was not guaranteed free of *Campylobacter*. Samples of liver, intestines and skin were obtained aseptically and prepared for extraction as described above. The tissue samples were transferred into a microcentrifuge tube and 200 µl of each bacterial dilution ranging from undiluted to a dilution of 10^{-6} was added. The extraction was then performed according to the protocol as described and each spiked sample and the pure bacterial dilutions were examined by PCR.

1.5 Selection of primers and semi-nested PCR

A slightly modified semi-nested PCR assay as described by Waage *et al.* (1999) was used in this study to detect *Campylobacter jejuni* and *Campylobacter coli*. Oligonucleotide primers from the *C. jejuni flaA* and *C. coli flaB* sequences with the following sequences were used: CF03-JT (5'-GCT CAA AGT GGT TCT TAT GC-3'), CF04-JT (5'-GCT GCG GAG TTC ATT CTA AGA CC-3') and CF02-JT (5'-AAG CAA GAA GTG TTC CAA GTT T-3'). The concentration of the primers were 76 pmol / µl for primer CF04-JT, 69 pmol / µl for primer CF02-JT and 79 pmol / µl for primer CF03-JT. The primers were obtained from Inqaba Biotech.

The first PCR step was performed with primers CF03-JT and CF04-JT and the resulting amplification was a fragment of 340 to 380 base-pairs (bp) as described by Waage *et al.* (1999). A total volume of 25 µl was used which contained Red Taq Ready Mix PCR reaction mix (12.50 µl), 0.25 µl of each primer, distilled water (9.50 µl) and the extracted DNA sample (2.50 µl).

The second PCR step was performed with the primers CF03-JT and CF02-JT and the resulting amplification was a fragment of 180 to 220 bp as described by Waage *et al.* (1999). A total volume of 25 µl was used and contained UDG (12.50 µl), 0.25 µl of each primer, water (11.50 µl) and 0.50 µl of PCR product of the first step.

The same PCR programme was used for both steps of the PCR. A pre-PCR step at 42°C for 2 min, heat denaturation at 94°C for 10 min, followed by 40 cycles consisting of heat denaturation at 94°C for 5 sec, primer annealing at 53°C for 30 sec and DNA extension at 72°C for 40 sec per cycle. After the last cycle, the samples were kept at 72°C for 10 min to complete synthesis of all strands and were kept at 4°C until analysed.

The PCR products were analysed on a 2% agarose gel, which was stained with ethidiumbromide. A volume of 10 µl of each final PCR product was loaded onto the gel and exposed to electrophoresis in 1xTris-Acidic acid – EDTA (TAE) buffer for 30 to 60

min at 130V. The DNA bands were visualized by UV illumination and identified against a 100-bp DNA ladder (Inqaba Biotech).

The **sensitivity** of the primers was tested by subjecting all bacterial dilutions as well as all tissue samples spiked with the range of bacterial dilutions to the PCR. Negative and positive controls were included into each PCR batch.

The **specificity** of the primers was determined by performing the PCR on the undiluted bacterial solution and tissue samples (liver, skin, intestines) of the SPF chicken. Sterile water was included in the PCR as negative control but it was not extracted with the samples.

2 Field study

To determine the status of *Campylobacter* in commercially available chickens, samples were taken at a fully mechanized, high-throughput South African poultry abattoir. The dates of sampling were chosen in a way to ensure that each batch of samples originated from a different farm supplying broilers to the abattoir. Fresh chicken carcasses were obtained randomly at the evisceration stage prior to chilling. Livers and intestines were obtained at the packaging stage at the abattoir prior to freezing in ready-to-sell packages. Samples were taken within a three week period in August/September (South African late winter season). A total number of 250 pooled skin samples (from 50 carcasses) and 25 samples of liver and intestines each were included in the study.

2.1 Poultry abattoir

The poultry abattoir where the samples were taken processes 5400 birds per line per hour on two lines. Birds are bled for 180 seconds and scalding is performed at a temperature of 50 – 52°C. The birds fall off the shackle onto a rubber transport belt after the hock and head cutting, and the carcasses are re-hung manually at the clean side of the processing line. Evisceration is partly mechanised in three steps whereby the carcasses are in close contact with the equipment. Viscera are loosened and lifted mechanically, but the final removal from the carcass is performed manually. After evisceration, the carcasses are spray-washed inside and outside with water containing 50-75 ppm of free chlorine.

Carcasses that are sold as fresh products undergo air-cooling for 45 minutes. Birds that are sold as frozen products are cooled in counterflow water spin-chillers for 25 min. The water consumption per bird is 2,5 liters, the water temperature is at 0–2° C and the chlorine content of the water is about 200 ppm. To improve the movement of the water

and the product buoyancy, air at environmental (room) temperature is injected into the spinchiller water from the bottom.

2.2 Sampling

Carcasses were obtained randomly post-evisceration and pre-chilling directly from the processing line. In the laboratory of the abattoir, which has a direct connection to the processing areas, 5 skin samples were taken aseptically from each carcass. The skin originated from the neck, both thighs and both sides of the breast. All skin pieces from one carcass were transferred into a small plastic bag as one pool sample per carcass. Each bag was consequently numbered and immediately placed on ice. Additional documentation ensured the identification of the individual sample number and the date and time the sample was taken.

After processing of the carcasses and skin samples, ready-to-sell packages of fresh intestines and liver were obtained at the packaging stage, prior to freezing (Figures 3.1 and 3.2). All samples were placed on ice and immediately transported to the laboratory for testing. The extraction process was started within 3 hours after collection.



Figure 3.1 Ready-to-sell packages of fresh intestines (mala)



Figure 3.2 Ready-to-sell packages of fresh livers

2.3 Extraction

The samples were processed in the laboratory as described above. To minimise the risk of cross-contamination, the weighing and cutting was performed aseptically. A total amount of 25 mg per sample was weighed, cut into very small pieces and placed into a microcentrifuge tube together with the prescribed buffer and Proteinase K. The samples were then vortexed briefly and incubated at 56°C for about 16 to 18 hours (overnight) to ensure complete lysis of tissue and bacterial cells. The extraction process was completed on the following day and the DNA was stored at minus 20°C until used for PCR.

2.4 PCR

To minimise the risk of cross-contamination, the PCR was performed in batches accordingly to the sampling dates. In the first PCR step, 2.5 µl of DNA was used, and the semi-nested, second PCR step, was performed using 0.5µl of the product resulting from the PCR in step 1. The PCR was performed as described above. Sterile water and SPF tissue sample DNA was used as negative controls and pure culture DNA as positive controls in the PCR with each batch.

2.5 Gel electrophoresis

A volume of 10 μ l of each final PCR product was loaded onto a 2% agarose gel that contained 2 μ l of ethidiumbromide. A 100-bp ladder was loaded onto each gel as reference. The gel was exposed to electrophoresis using a 1xTAE buffer at 130V for 30 to 60 minutes. The results were visualized under UV illumination and photographs were taken and stored electronically. The Kodak EDAC gel documentation system (Laboratory Specialist Services) was used.

CHAPTER 4

Results

1 Pilot study

1.1 Results

1.1.1 Specificity of primers

The specificity of the primers was determined by performing the PCR on the undiluted bacterial solution and tissue samples (liver, skin, intestines) of a SPF chicken. Sterile water was not processed together with the extraction of samples, but included in the PCR as a negative control. The semi-nested PCR step resulted in a fragment of the expected size of 180 to 220 bp for the undiluted bacterial culture, while the SPF chicken tissue samples did not show a DNA band.

1.1.2 Sensitivity of primers

The sensitivity of the primers was tested by subjecting all bacterial dilutions as well as all tissue samples spiked with the range of bacterial dilutions, to the PCR. Negative (unspiked SPF tissue samples) and positive (pure bacterial culture) controls were included into each batch of PCR. All tissue samples taken from the SPF chicken were negative in the PCR. The undiluted culture solution, as well as all tissue samples spiked therewith, showed DNA bands of the expected size. The results are summarised in Table 4.1.

Table 4.1 Results of PCR on the SPF chicken samples to test the sensitivity and specificity of primers used

Sample	Not spiked	Tissue samples spiked with:						
		Pure culture	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
SPF liver	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
SPF skin	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
SPF intestines	(-)	(+)	(+)	(+)	(+)	(+)	(-)*	(+)
Culture dilutions	Not applicable	(+)	(+)	(+)	(+)	(+)	(+)	(+)

The PCR performed on the culture dilutions and the spiked tissue samples showed clearly positive results, with the exception of the SPF intestines solution 10^{-5} as highlighted in grey.

2 Field study

2.1 Results

Samples were taken at a high throughput commercial South African poultry abattoir over a period of three weeks in late winter. To avoid the risk of cross-contamination, extraction and PCR was performed in batches (Tables 4.2 to 4.4). Each batch contained 5 liver, 5 intestine and 10 skin samples.

Table 4.2 Results of PCR performed on liver samples obtained at the abattoir

Liver	Result	Batch
1	(+)	1
2	(-)	1
3	(-)	1
4	(-)	1
5	(+)	1
6	(-)	2
7	(-)	2
8	(+)	2
9	(+)	2
10	(+)	2
11	(+)	3
12	(-)	3
13	(-)	3
14	(-)	3
15	(-)	3
16	(-)	4
17	(-)	4
18	(-)	4
19	(-)	4
20	(-)	4
21	(-)	5
22	(-)	5
23	(-)	5
24	(-)	5
25	(-)	5

Table 4.3 Results of PCR performed on samples of intestines obtained at the abattoir

Intestines	Result	Batch
1	(-)	1
2	(-)	1
3	(-)	1
4	(-)	1
5	(-)	1
6	(+)	2
7	(-)	2
8	(-)	2
9	(-)	2
10	(+)	2
11	(-)	3
12	(-)	3
13	(-)	3
14	(+)	3
15	(-)	3
16	(-)	4
17	(-)	4
18	(-)	4
19	(-)	4
20	(-)	4
21	(-)	5
22	(+)	5
23	(+)	5
24	(+)	5
25	(+)	5

Table 4.4 Results of PCR performed on skin samples obtained at the abattoir

Skin	Result	Batch
1	(+)	1
2	(+)	1
3	(+)	1
4	(+)	1
5	(+)	1
6	(+)	1
7	(+)	1
8	(-)	1
9	(-)	1
10	(-)	1
11	(-)	2
12	(+)	2
13	(-)	2
14	(-)	2
15	(-)	2
16	(+)	2
17	(-)	2
18	(-)	2
19	(-)	2
20	(+)	2
21	(-)	3
22	(-)	3
23	(-)	3
24	(-)	3
25	(+)	3

Skin	Result	Batch
26	(-)	3
27	(-)	3
28	(-)	3
29	(-)	3
30	(-)	3
31	(-)	4
32	(-)	4
33	(-)	4
34	(-)	4
35	(-)	4
36	(-)	4
37	(-)	4
38	(-)	4
39	(-)	4
40	(-)	4
41	(-)	5
42	(-)	5
43	(-)	5
44	(-)	5
45	(-)	5
46	(-)	5
47	(+)	5
48	(-)	5
49	(-)	5
50	(-)	5

With respect to the correlation of contamination rates of different tissues within the same batch, the following was observed.

- The first batch showed a high contamination of skin (70%) and a moderate contamination of liver samples (40%), while all intestine samples were negative.
- The second batch was more homogenous, with 60%, 40% and 30% for liver, intestines and skin, respectively.
- The same applied to batch number 3, with 20% positive samples each for liver and intestines, and 10% positive samples for skin.
- While all tissue samples of the fourth batch were negative, 80% of all intestine samples in batch number 5 showed positive results, but all liver and skin samples out of this batch were negative. These results are summarised in Table 4.5 and illustrated in Figures 4.1 and 4.2.

Table 4.5 Results of PCR performed on field samples, listed according to batches

Sample	Liver		Intestines		Skin	
	(5 per batch)		(5 per batch)		(10 per batch)	
	Positive	Positive	Positive	Positive	Positive	Positive
	(Total)	%	(Total)	%	(Total)	%
Batch 1	2	40%	0		7	70%
Batch 2	3	60%	2	40%	3	30%
Batch 3	1	20%	1	20%	1	10%
Batch 4	0		0		0	
Batch 5	0		4	80%	0	

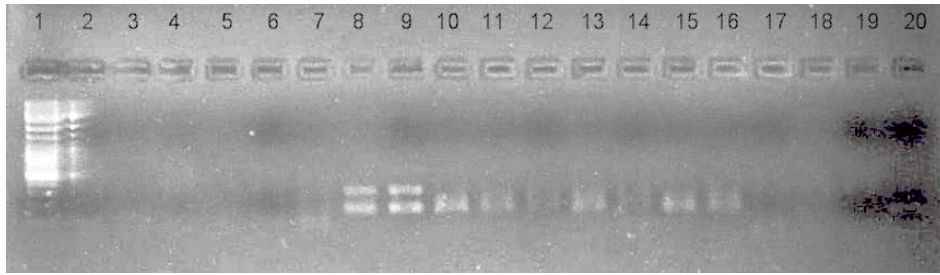


Figure 4.1 PCR results of skin samples taken as the first batch

1. DNA ladder
6. SPF skin (negative control)
7. H₂O (negative control)
8. SPF skin spiked with bacterial culture (positive control)
9. pure bacterial culture (positive control)
10. skin sample 1
11. skin sample 2
12. skin sample 3
13. skin sample 4
14. skin sample 5
15. skin sample 6
16. skin sample 7
17. skin sample 8
18. skin sample 9
19. skin sample 10
20. H₂O (2nd step PCR, negative control)

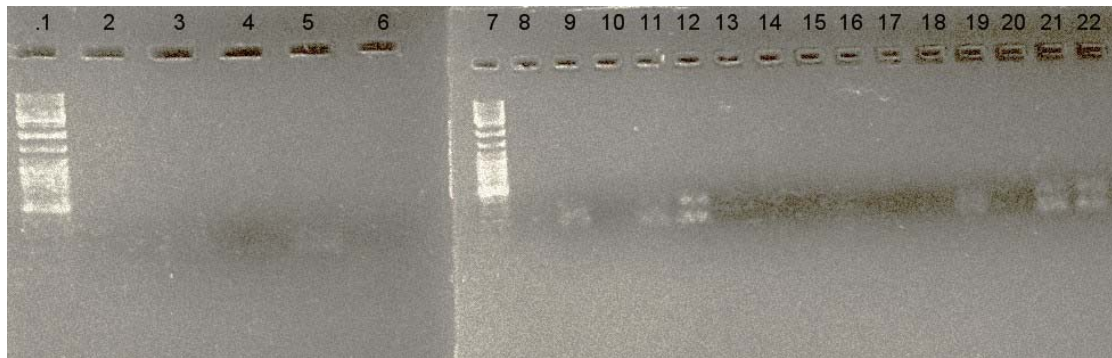


Figure 4.2 PCR results of liver and intestine (mala) samples taken as the third batch

1. DNA ladder
2. SPF liver (negative control)
3. H₂O (negative control)
4. H₂O (2nd step PCR, negative control)
5. liver sample 11
6. liver sample 12
7. DNA ladder
8. liver sample 13
9. liver sample 14
10. liver sample 15
11. SPF liver spiked with bacterial culture (positive control)
12. pure bacterial culture (positive control)
13. SPF intestines (negative control)
14. H₂O (negative control)
15. H₂O (2nd step PCR, negative control)
16. intestine (mala) sample 11
17. intestine sample 12
18. intestine sample 13
19. intestine sample 14
20. intestine sample 15
21. SPF intestines spiked with bacterial culture (positive control)
22. pure bacterial culture (positive control)

In conclusion, the liver and the skin samples show the same average contamination rate (24%) with *Campylobacter*. A total of 6 samples out of 25 samples of the liver tissue were positive, and 12 out of 50 pooled samples of skin revealed positive PCR results. The intestine samples showed a slightly higher rate of *Campylobacter* spp.

contamination, with 7 out of 25 samples or 28%. These results are demonstrated in Table 4.6 and in Figure 4.3.

Table 4.6 Field samples: Summary of results

Sample	Total	Positive (Total)	Positive (%)	Negative (Total)	Negative (%)
Liver	25	6	24%	19	76%
Intestines	25	7	28%	18	72%
Skin (pooled samples)	50	12	24%	38	76%

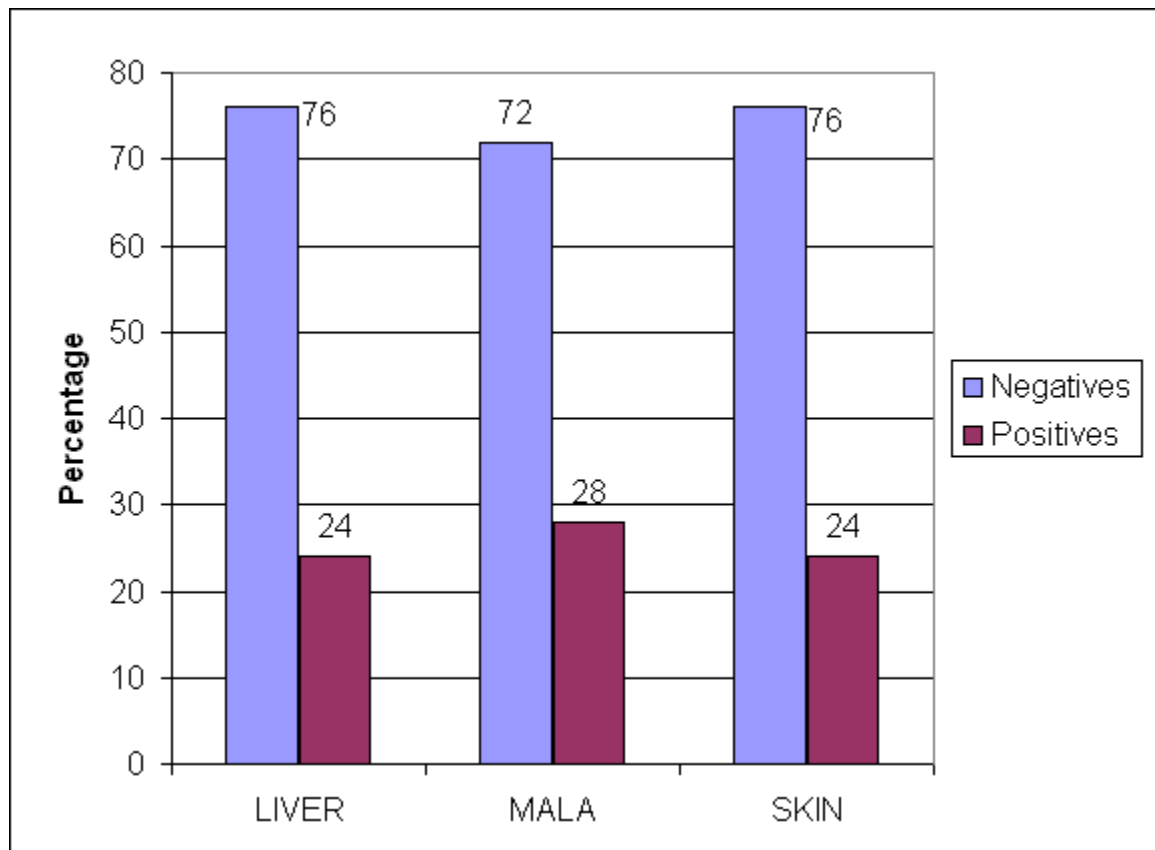


Figure 4.3 Results of PCR performed on the field samples expressed as a percentage

CHAPTER 5

Discussion and conclusions

1 Discussion

1.1 Pilot study

The pilot study of this research project aimed at the determination of specificity and sensitivity of primers and method for the detection of *Campylobacter*. It revealed that primers and methods chosen were appropriate and specific to detect the organism in bacterial solutions and in tissue samples.

As the tissue samples used varied in terms of composition and possible bacterial contamination, an extraction method suitable for all three types of tissue had to be used. Extraction by boiling did not produce sufficient lysis of the samples. Therefore, the tissue protocol as described in the QIAamp DNA Mini Kit Manual was used. However, the prescribed time for incubating the samples with Proteinase K was insufficient and the tissue was not lysed completely. Therefore we incubated all samples overnight. With this modification the extraction method used resulted in the complete lysis of each tissue sample tested.

The primers were chosen in accordance to the protocol as described by Waage *et al.*, (1999), as these researchers used those primers successfully with a variety of food and water samples. Our PCR assay resulted in amplifications similar to those described by Waage *et al.* (1999) in both steps of the PCR.

The first step PCR showed bands of the expected size and these results were clearly confirmed with the second, semi-nested PCR step. This indicates that the semi-nested PCR is a more accurate and specific method to detect *Campylobacter* although it can lead to more contamination of the PCR products. Only one of all spiked samples tested was negative. The intestine sample spiked with a bacterial dilution of 10^{-5} had a negative result using the PCR. This is most probably caused by a technical problem during the course of the laboratory procedures as the next dilution of 10^{-6} showed a positive PCR result.

The results of the pilot study revealed a high sensitivity of the primers and method, which enabled the detection of DNA equivalent to 58 bacterial cells per ml or 12 cells per PCR, based on the results for a bacterial dilution of 10^{-6} . This is congruent to similar assays

that are described by Mandrell and Wachtel (1999) with detection rates of 35 to 120 *Campylobacter* cells per ml.

1.2 Field study

The field study was performed on a total number of 300 tissue samples (skin, livers, intestines). The samples were obtained randomly at the post-evisceration stage of the processing line of a high throughput poultry abattoir and as ready-to-sell packages prior to freezing. All samples were tested for the presence of *Campylobacter* spp. by a semi-nested PCR assay. The sampling site at the processing line was chosen because the evisceration stage prior to chilling is regarded as one of the most critical points with regard to the risk of cross-contamination during the processing of poultry (Li *et al.*, 1995; Kemp and Schneider, 2002).

The different tissues included in the study were chosen according to the predilection sites of *Campylobacter* in poultry as described by various researchers (Oosterom *et al.*, 1983; Atanassova and Ring, 1999; Shih, 2000) and with regard to the nutritional importance of the different products.

As an enteric pathogen, *Campylobacter* is commonly found in the intestinal flora of poultry and carcass contamination is common during processing (Beery *et al.*, 1988; Whyte *et al.*, 2001). Furthermore, livers and intestines of poultry form an important part of the traditional diet in the African population (Ditshwantsho tsa Rona, 1983). While livers have been the subject of various research papers, intestines as an important edible poultry product have not been addressed in previous studies (Table 1.2). This study therefore closes a gap of importance for the African situation by including intestines into this research.

As many authors described the skin of neck, breast and thighs as the predilection sites for *Campylobacter* on the chicken carcass, those sites were sampled for this study (Berndtson *et al.*, 1992; Kotula and Pandya, 1995; Berrang and Buhr, 2001). Thomas and McMeekin (1980) described the topography of poultry skin with regard to contamination with microorganisms. According to this study, organisms are partly trapped in feather follicles, channels and folds of skin of carcasses or products and therefore not readily removable. Subsequently, surface swabs and washes might not include all bacteria present on the carcass. Based on these findings it was decided that lysed samples of tissue should be used for this study rather than washes or swabs to ensure that all bacteria present trapped in tissue folds and attached to the surface would be detected. Five skin samples per carcass were obtained from the sites mentioned and processed as a pooled sample.

A large majority of studies is based on the use of carcass washes or rinses and includes an enrichment stage before the further processing and examination of the samples. Our approach to detect *Campylobacter* was different with the use of solid tissue samples and direct processing of samples. This was done to avoid the possibility that substances present in the enrichment media could inhibit the PCR. Furthermore, we wanted to limit the time necessary to complete the assay. Even with the prolonged time necessary for tissue lysis during the extraction stage, the examination of tissue samples could be completed in about 28 hours, from the sampling to the visualization of the PCR product.

Secondly, we chose a semi-nested PCR assay instead of a single step PCR as the semi-nested method is more sensitive and aims more specifically at the target DNA, and also excludes contaminating DNA.

A number of studies on *Campylobacter* contamination of poultry products have been performed over the past 20 years (Table 1.2). In these studies, contamination rates varying from 0% to 95% were reported. Our findings correspond with these results. A precise evaluation of our results in comparison with the findings cited in many of the studies mentioned is, however, not possible as information regarding the season of sampling is often not provided. The prevalence of *Campylobacter* in poultry flocks and subsequently in poultry products is closely related to the various climatic conditions of a specific season, and any information pertaining to the season and climatic conditions at sampling time is regarded as important for the evaluation of the contamination rates.

2 Conclusions

The two main objectives of the study presented were

- To determine the extent of the contamination of poultry products with *Campylobacter jejuni* and *Campylobacter coli* in a high throughput South African chicken abattoir, and
- To develop a convenient and practical method for identifying *Campylobacter jejuni* and *Campylobacter coli* in the obtained samples.

Findings similar to those published by numerous authors as cited in the literature review were expected as an outcome of this study (Oosterom *et al.*, 1983; Berndtson *et al.*, 1992; Giesendorf *et al.*, 1992; Kotula and Pandya, 1995; Aquino *et al.*, 1996; Shih, 2000; Meldrum *et al.*, 2004).

This study will benefit consumers, the public health sector and the poultry industry in South Africa, as it will give a first indication about the prevalence of *Campylobacter* in poultry meat and products processed in a mainly mechanised chicken abattoir. Furthermore, it will form a basis for further investigation. The obtained information about the prevalence and the distribution of *Campylobacter* in chicken meat and products will be useful for the implementation of control methods such as a hazard analysis of critical control point (HACCP) food safety management system to minimise the public health risk of *Campylobacter* enteritis in South Africa.

Campylobacteriosis in humans is the leading cause of acute bacterial diarrhoea in many developed and developing countries. While extensive research has resulted in valuable data regarding the prevalence and epidemiology of *Campylobacter* as a food borne zoonosis in developed countries during the past 20 years, similar information from developing countries is very limited due to a lack of national surveillance programmes and research projects in these countries (Anderson *et al.*, 2003; Alter *et al.*, 2005).

The hypothesis stated for this study was that *Campylobacter* would be present in samples of chicken meat and products obtained at a high throughput poultry abattoir in South Africa. This was confirmed by the results as described above.

The findings are in line with those of other publications considering the season during which the samples were obtained. Lower contamination rates were expected as sampling was performed in late winter (dry season) in a summer rainfall area in South Africa. Contamination rates are high in summer and autumn and isolation of *Campylobacter* is more frequently reported in wet or humid climatic conditions. The lowest incidence is reported to be in late winter and early spring and under dry conditions (Blaser *et al.*, 1979; Blaser *et al.*, 1983; Skirrow, 1991; Jacob-Reitsma *et al.*, 1994, Stern, 1995; Berndtson *et al.*, 1996b; Willis and Murray, 1997; Atanassova and Ring, 1999).

Chicken meat and chicken products form an important part of the traditional diet in the African population. This study closes an information gap of importance for the African situation by including intestines into the research.

Human campylobacteriosis is an important food borne zoonosis. The handling of raw chicken products in the household bears high risks of cross-contamination and infection for consumers (Harris *et al.*, 1986; Skirrow, 1991; Lee *et al.*, 1998). In the African context, these risk factors for the transmission of *Campylobacter* cannot be overemphasized. Chicken and chicken products form a substantial part of the traditional diet, as they are cheap and easily available outside of supermarkets and other retail

outlets. Street vendors and hawkers who do not have cooling facilities commonly sell especially livers and intestines. The products are usually obtained at abattoirs and butchers by the hawkers, and sold in the streets during the same day, displayed on tables or in cartons at environment temperatures (Ditshwantsho tsa Rona, 1983). The break in the cold chain, especially under South African climatic conditions, favours the multiplication and consequently the increase of numbers of *Campylobacter* bacteria already present in the chicken meat and products. Furthermore, street vendors and hawkers do not have readily accessible hand washing facilities and will consequently disseminate the bacteria via their contaminated hands to other products. The subsequent handling of such products in households and the potential for cross-contamination of other foods therefore presents a high risk of infection to consumers.

Conventional detection of *Campylobacter* in food depends on selective cultural enrichment followed by isolation from selective agar. Identification and confirmation is based on biochemical tests. These methods are time consuming and laborious and require an average time of 4 – 6 days.

DNA hybridization and PCR have been developed as a rapid, sensitive and reliable alternative to detect *Campylobacter* in food samples. This method allows first results within 48 hours. Several PCR assays, with and without pre-enrichment, have been described in literature (Giesendorf *et al.*, 1992; Hazeleger *et al.*, 1994; Winters and Slavik, 1995; Docherty *et al.*, 1996; Ng *et al.*, 1997; Waage *et al.*, 1999; Thunberg *et al.*, 2000).

For this study we used a PCR method that is fast and sensitive. Solid tissue samples for the DNA extraction were used instead of tissue rinses or washes and no enrichment step was performed. This reduced the time necessary to complete the test to 28 hours. In order to increase the specificity and the sensitivity of the test, the PCR was performed in two steps. However, a nested or semi-nested PCR has the disadvantage that it can lead to more contamination during the processing of samples. As little as 58 bacteria per ml of the tissue extract or 12 bacteria per PCR could be detected by the method described.

In comparison with other methods described by various authors this method is fast and sensitive and will therefore be suitable for the screening of large numbers of samples.

Further investigations are necessary on farm level to determine the status of flock colonisation in South Africa. The processing and retail level should be investigated to quantify the risk for consumers to contract the infection via poultry products. It would also be advisable to extend the sampling periods over one year to obtain reliable data

regarding the seasonal trends in the incidence of *Campylobacter* infections in South Africa.

Human campylobacteriosis poses a high risk for immuno-compromised individuals. To help understand the extent of the problem, a screening of patients by medical doctors and hospitals to reveal the incidence of human campylobacteriosis and subclinical infection is needed.

This study should be considered as a basis for further research. Depending on the results obtained from further research as mentioned above, appropriate control measures might need to be introduced.

Intervention measures on farm level in order to reduce the initial bacterial load of poultry entering the processing plants have so far proven to be of limited effect (Kemp and Schneider, 2002). Therefore, decontamination procedures within processing and retailing facilities and the information and education of consumers on the importance of hygiene in the kitchen and during food handling will remain the primary line of defence to eradicate *Campylobacter* from poultry products and to decrease the incidence of human campylobacteriosis (Harris *et al.*, 1986, WHO, 2000; Rosenquist *et al.*, 2003).

In addition, emphasis should be placed on the poultry industry on farm level and in the post-harvesting phase. Good management practices on the farm, including the use of an all-in-all-out-system with proper cleanout and disinfection between the flocks are effective measures to considerably reduce the colonization of a flock reducing the initial bacterial load of broilers arriving at the abattoirs (Hoop and Ehram, 1987; Jacobs-Reitsma *et al.*, 1994; Jacobs-Reitsma, 1997; Saleha *et al.*, 1997; Beery *et al.*, 1988; Evans and Sayers, 2000). During processing, risk assessment models for the facilities and the introduction of HACCP programmes are essential measures to reduce the risk of cross-contamination of *Campylobacter* (Anderson *et al.*, 2003; Rosenquist *et al.*, 2003).

In conclusion this study proves that *Campylobacter* are prevalent in poultry in South Africa and that the contamination of poultry meat and products with this organism could present a health hazard for consumers and hence further investigation and the application of appropriate control measures are needed.

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