Prevalence and characterisation of *Campylobacter* species from chickens sold in informal poultry markets in Gauteng, South Africa

By

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Declaration

I hereby declare that this dissertation titled “Prevalence and characterisation of *Campylobacter* species from chickens sold in informal poultry markets in Gauteng, South Africa” is my own work and no part of it has been previously submitted for any degree at this or any university.

2018/12/07

Signature Date

Dedication

To my dearest mother Melidah Phosa and my late father Masiapata Phosa, I dedicate this work to you. You sacrificed so much for us to be educated. Thank you, your sacrifices were not in vain!

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Abbreviations

≈	Approximately
<	Less than
=	Equal to
>	Greater than
°C	Degree Celsius
ml	Millilitre
µl	Microlitre
µM	Micromolar
5'-3'	Five prime to three prime direction
%	Percentage
AEC	Animal Ethics Committee
ATM	Amies Transport Medium
ATCC	American Type Culture Collection
AgriSETA	Agriculture sector education training authority
Asp	Aspartokinase gene
bp	Base pairs
BHI	Brain Heart Infusion
BB	Bolton Broth
BR	Broad range
BPW	Buffered peptone water
BA	Butzler Agar
CO_2	Carbon Dioxide
C. coli	*Campylobacter coli*

C. jejuni	*Campylobacter jejuni*
CLSI	Clinical and laboratory standards institution
CCDA	Charcoal Cefoperazone Deoxychocolate agar
CDC	Centre for Disease Control
CF	Complement fixation
CDT	Cytolethal distending toxin
DNA	Deoxyribonucleic Acid
DAFF	Department of Agricultural and Forestry Fisheries
DVTD	Department of Veterinary Tropical Diseases
EIA	Enzyme immunoassay
GIT	Gastrointestinal Tract
GDARD	Gauteng Department of Agriculture and Rural Development
GPS	Global positioning system
GBS	Guillain-Barré syndrome
HACCP	Hazard analysis critical control point
hipO	Hippurate Hydrolysis gene
HIV	Human Immunodeficiency Virus
h	Hour/s
IFA	Immunofluorescent antibody
IBM	International Business Machine Corporations
ISO	International Standard of Operations
kb	Kilobase/s
Km	Kilometres
MIC	Minimum inhibitory concentration

MFS	Miller Fisher Syndrome
min	Minute/s
mPCR	Multiplex polymerase chain reaction
NAMC	National Agricultural Marketing Council
NRF	National Research Foundation
N_2	Nitrogen
OR	Odds ratio
O_2	Oxygen
OVR-ARC	Onderstepoort Veterinary Research-Agricultural Research Council
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PA	Preston Agar
ReA	Reactive Arthritis
RNA	Ribonucleic acid
RT-PCR	Real Time-Polymerase Chain Reaction
rpm	Revolutions per minute
SPSS	Statistical Package for the Social Sciences
s	Second/s
SAPA	South African Poultry Association
UP	University of Pretoria
VBNC	Viable but non-culturable
WHO	World Health Organisation
g	Gravitational force

Dissertation Summary

Prevalence and characterisation of *Campylobacter* species from chickens sold in informal poultry markets in Gauteng, South Africa

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Consumption of chicken is high compared to other meat products in South Africa and this has resulted in increased demand for chicken and growth of the informal poultry market, which is unregulated by appropriate government agencies. It is generally believed that the quality of chickens bought from the informal outlets is poor and may pose health hazards to consumers. Unsanitary practices at the informal market will allow opportunist pathogens such as *Campylobacter* species to contaminate the chicken products which could therefore cause Campylobacteriosis in humans. Campylobacteriosis is foodborne disease which is associated with consumption of *Campylobacter* contaminated meat products.

The aim of the study was to determine the prevalence, risk factors and characteristics of *Campylobacter* species contamination in chicken carcasses sold at informal poultry outlets in Gauteng province, South Africa. The study was conducted in six areas (Germiston, Atteridgeville/Phomolong, Garankuwa, Tembisa/Modise, Alexandra, Soweto) from which a total of 151 chicken carcasses were collected across 47 outlets. From each chicken carcass the carcass drip, cloacal and carcass swabs were sampled which resulted in a total of 453 samples collected. At each outlet, a questionnaire was administered to owners. *Campylobacter* species were isolated from the samples using conventional bacteriological methods and isolates were confirmed by conventional PCR and speciated into *C. coli* and *C. jejuni*. Broth cultures were screened for genus identification of *Campylobacter* species and speciation into *C. coli* and *C. jejuni* using conventional PCR. Six virulence genes (*ciaB, dnaj, pldA, racR, flaA, flaB*), three toxin genes (*cdtA, cdtB, cdtC*) and one antimicrobial resistance gene *(tetO)* were screened by using duplex and multiplex PCR.

From the 47 identified outlets, differences were observed in their processing methods, with majority of outlets storing their live chickens in cages, using knives to slaughter the chickens and eviscerating chickens after slaughter. Of all the seven processing parameters, culled breeders (OR = 9.7), followed by spent hens (OR = 7.2), stagnant water (OR = 2.0) and defeathering (OR = 1.8) were significantly higher. From the four risk factor parameters, only poor sanitation (OR = 2.5) was significantly higher.

The frequency of isolation of *Campylobacter* species from carcass drip, cloacal and carcass swabs using standard bacteriology methods was 40.4% (P = 0.0443), 37.7% (P = 0.0235) and 24.5% (P = 0.0091) was statistically significant respectively across the three types of samples. The cloaca is the normal habitat for resident *Campylobacter*. However, the highest frequency of isolation of *Campylobacter* species was detected in carcass drip (40.4%, P = 0.0443). This could be explained, in part, by contamination from rinse water, processing and sale tables, human handling and knives used at these outlets. The frequency of isolation of *Campylobacter* species from carcass samples was the highest in Germiston (66.7%) and lowest in Tembisa/Modise (0.0%). Processing methods and practices in these two areas were different which led to different levels of *Campylobacter* isolation. Informal chicken outlets in Germiston were slaughtering more culled breeders (36.7%) and spent hens (55.0%) while outlets

in Tembisa/Modise slaughtered more broilers (70.0%). The rinsing method was also different, with the outlets in Germiston rinsing from stagnant water in buckets (100.0%) and the outlets in Tembisa/Modise rinsing from stagnant water in drums (100.0%). The water used in Germiston was easily saturated increasing the risk of cross contamination. The total frequency of *Campylobacter* species using standard bacteriology was 34.2% (P = 0.0201) (155/453 isolates). Conventional PCR confirmed 68.4% (P = 0.0276) as true *Campylobacters* and 6.6% was speciated into *C. coli* and 6.6% was *C. jejuni* and the remaining were not speciated.

The frequency of virulence and toxin genes was higher in *C. jejuni* (69.0%, P = 0.0784) positive broth cultures than in *C. coli* (38.7%, P = 0.0497) positive broth cultures however, the frequency of virulence genes was only statistically significant in *C. coli*-positive BB (0.0497). This could be explained in part by the number of human campylobacteriosis cases which are linked to *C. jejuni*. The frequency of tetracycline resistance gene *(tetO)* was higher in *C. coli* (68.3%) than in *C. jejuni* (50.0%). It is concluded that the high frequency of virulence, toxin and antibiotic resistance genes in the *Campylobacter jejuni* recovered from chickens slaughtered at the informal chicken market in Gauteng province may pose food safety and therapeutic concerns to consumers of inadequately cooked chickens from these outlets. The high prevalence of *Campylobacter* species in chicken samples studied could be reduced by the elimination of the risk factors identified for the contamination of chickens during processing.

Chapter 1: Introduction

1.1 Introduction

The informal poultry market of South Africa is a highly patronized market which can be entered by any individual from different walks of life. In its unorganised state it has found a way to sustain itself because the demand for chicken and chicken products in South Africa is very high. Most South Africans consume chicken which has high protein and is very affordable. The economic history of this country has allowed this type of market to thrive, whereby many South Africans and foreign residents can easily get into this business to support their families. The problem with an 'industry' that is unregulated is that it can be vulnerable for abuse due to no clear guidelines for the operation and thus, allowing people to do as they see fit. In the food industry, from farm to retail standard guidelines do exist for food safety and security in order to protect both the consumer and food handlers.

In the informal poultry market there are no regulations or monitoring processes. Thus, there is no information on what happens during processing which includes defeathering, rinsing and packaging. It is important have a detailed understanding of the whole operation since poultry meat is a possible carrier of foodborne pathogens such as *E. coli, Salmonella* species, *Staphylococcus aureus* and *Campylobacter,* in order to reduce the risk of contamination of chickens during processing. In this study we focused on *Campylobacter* species, which is a Gram-negative, spirally curved rod which grows under microaerophilic conditions and can withstand high temperatures ranging from 37^{o}C – 42^{o}C (Vandamme and De Ley, 1991).

Campylobacteriosis is an infection caused by *Campylobacter* species (WHO, 2015). Campylobacteriosis, is a foodborne disease and therefore a disease of major public importance caused by *C. coli* and *C. jejuni*. More than 90% of gastrointestinal campylobacteriosis cases have been linked to *C. coli* and *C. jejuni* as the source of infection (Wagenaar et al., 2014).

Campylobacteriosis in humans has mild clinical signs with an incubation period of two to five days. Symptoms vary but the most common are watery or sticky diarrhoea, abdominal cramps, fever, nausea, vomiting, muscle pain and bloody faeces

(Mackenjee et al., 1984; Shobo et al., 2016). Campylobacteriosis is a self-limiting disease which helps the recovery phase because the infection only lasts for three to five days and complications are rare.

Antibiotics are rarely administered for this infection and in cases of severe gastroenteritis where the infection is prolonged, antibiotics are prescribed for treatment (Levin, 2007). Miller Fisher syndrome (MFS) and Guillain-Barré syndrome (GBS) are two autoimmune illnesses related to campylobacteriosis and are usually observed within 10 days after infection. The drugs of choice for severe gastroenteritis are macrolides, fluoroquinolones and tetracyclines. These drugs are not only used in human campylobacteriosis, they are added to feed in poultry and used as prophylaxis and growth promoters (Moyane et al., 2013). The emergence of *Campylobacter* resistant strains is a public health concern because if an individual is infected with a resistant strain treatment drugs will be limited (Reddy and Zishiri, 2017).

Worldwide, the rate of human *Campylobacter* infections is higher than that of *Salmonella, Yersinia* and *E. coli* combined (Acheson and Allos, 2001). Therefore, it is prudent to know the prevalence of *Campylobacter* species in its natural habitat and the prevalence of both *C. coli* and *C. jejuni,* as they are the most frequently isolated species in in the avian gastrointestinal tract (GIT). It is also important to understand the pathogenesis and if isolates are resistant or susceptible to the antibiotics which are used in humans and animals.

1.2 South African poultry market

Majority of South Africans prefer to consume poultry meat because it has high protein content, it is a good source of vitamins and compared to other meat, poultry meat is affordable. This has intensified the growth of the informal poultry market because there is a high demand for poultry meat which cannot be met by only the formal retail market. The informal poultry market in South Africa is an open market which can be frequented by people from different walks of life. In the country, the informal poultry market is an open market which can be started by educated or uneducated South Africans and foreigners. Start up cash and a suitable spot where people can see the vendor is enough to start the business.

In South Africa, poultry farming is part of the agriculture industry which contributes a total of 15.2% of the 46.9% gross value of animal products and is thus a significant player in the economy of the country. In South Africa there are many factors affecting the poultry industry, such as climate change, land quality and policies governing tenure. Climactic changes, such as temperature and precipitation can have a negative effect on the health of the animals due to changes in diet and or diseases caused by parasites whose presence in the environment can be dictated by climate (Godfray et al., 2010).

There has been an increase in the population of South African between 2013 – 2018, from 53.1 – 56.5 million and the current unemployment rate is 27.2% (Statistics SA, 2018). Due to increase in inflation rates and taxes, meat is not affordable for the majority of South Africans due to unemployment and low wages (Fine, 2018). According to the National Agricultural Marketing Council (NAMC, 2015) of South Africa, the price of pork chops is R 68/kg, beef mince R 65.49/kg and beef (T-bone) at R 82.67/kg, the most expensive. Poultry and its products are much more affordable as compared to the above-mentioned meat prices. Whole chicken is currently sold at R 39.50/kg and chicken portions at R 49.85/kg.

It makes financial sense for many South Africans, especially the poor to choose poultry meat as a source of protein. This in turn increases the demand for poultry and poultry products, which has encouraged a growth in the informal poultry market. These markets are usually unregulated and often based in rural or poor communities.

Different industries are governed by different sets of guidelines that help to minimise abuse such as the, International Standard of Operations (ISO) which have been developed and accustomed for different industries. There are ISO standards which govern the food industries to ensure consumption of safe food and to minimise disease outbreaks (Grindley, 1995). There are regulations/guidelines that govern the commercial farmers, abattoirs and retail markets (Alston and Scobie, 1987). The informal poultry markets are not regulated and consequently do not operate using ISO standards and thus do not ensure the consumption of safe food. This in turn can result in food-borne diseases in communities where contaminated products are purchased and consumed.

Therefore, consumption of poultry and poultry products from informal markets is a public health concern because there are no slaughtering and processing regulations that protect the consumer from buying contaminated chickens. The consumer, without any knowledge of food safety, is susceptible to infection with *C. coli* or *C. jejuni* in particular if the poultry is not cooked adequately before consumption.

Chapter 2: Literature Review

2.1 Campylobacteriosis

According to the World Health Organisation (WHO) a foodborne disease results from the consumption of food contaminated with chemicals or microorganisms such as bacteria, viruses and fungi. Foodborne diseases can be viral, bacterial or fungal (WHO, 2015). Campylobacteriosis is a foodborne disease and poultry is a major culprit in the spread of this disease (Kapperud et al., 1992).

Campylobacteriosis is a foodborne disease because the aetiological agent is shed in the faeces of the animal and contaminates meat products such as poultry, beef, lamb and pork (Coker et al., 2002). Although *Campylobacter* can colonize the intestinal tract of most food producing animals, it is most likely to colonize the intestinal tract of avian species (Adak et al., 2005). Chicken carcasses can be contaminated with faecal matter during slaughter making the animal products a source for human infections. Although *Campylobacter* species are commensals to many warm-blooded animals, transition from being normal resident microbiota to pathogenic has not yet been clearly elucidated (Coker et al., 2002).

In the European Union (EU) report, campylobacteriosis is the leading zoonotic disease followed by salmonellosis and yersinosis and this finding was consistent with a paper published by Acheson and Allos, (2001) where they found that in more developed countries the number of *Campylobacter* infections were higher than those of *Salmonella*, *Shigella and Escherichia coli* O157:H7 infections combined.

There are insufficient data on campylobacteriosis in Africa due to the lack of resources. In South Africa current literature focuses mostly on clinical cases and the detection of *Campylobacter* in water (Obi et al., 2002) and in products sold at the formal/retail food markets (Jonker and Picard, 2010, Otigbu et al., 2018). In South Africa cases of campylobacteriosis are under-reported due to lack of surveillance and the fact that this is a self-limiting illness which can end without any administration of drugs.

The form of campylobacteriosis of major importance is gastroenteritis caused by *C. coli* and *C. jejuni* (Coker et al., 2002). These two species of *Campylobacter* have been isolated from human cases in both developing and developed countries and the

prevalence of *C. jejuni* isolates associated with human disease exceeds that of *C. coli.* Campylobacteriosis is a self-limiting disease and therefore, it is rare to find complications associated with the disease most especially in individuals who are not immunocompromised or under the age of five years because the pathogen cannot survive longer in the host (Coker et al., 2002, Acheson and Allos, 2001, Bester and Essack, 2008, Cantero et al., 2018).

Complications related to *Campylobacter* infections become apparent a week or a month after the initial infection. Therefore, *Campylobacter* species cannot be detected in stools which leaves the stools with viable and non-culturable (VBNC) *Campylobacter* species (Ang et al., 2007). VBNC's can only be detected by molecular techniques or serological assays. Molecular techniques will screen for DNA and serology detects antibodies which were released in response to the pathogen (Ang et al., 2007). Both of these techniques do not confirm the presence of a pathogen; however, they only indicate that there was exposure to *Campylobacter* by either presence of DNA in PCR or antibodies in serology.

These post-infection complications can be grouped into two categories. The extra intestinal complications are Reactive Arthritis (ReA), pancreatitis and endocarditis. The neurological diseases are MFS and GBS (Ang et al., 2007, Janvier et al., 2000). GBS is an autoimmune disorder of the peripheral nervous system, characterised by flaccid paralysis, which is now recognized as a post-infectious complication of *C. jejuni* infection. However, its incidence is less than one per 1000 cases of campylobacteriosis (Coker et al., 2002, Silva et al., 2011).

2.2 *Campylobacter* species

Campylobacter species are from the family of Campylobacteraceae which are grouped into three genera: *Campylobacter, Arcobacter* and *Sulfurospirillum* (Lastovica et al., 2014). There are approximately 30 species of *Campylobacter* and 16 sub-species (Lastovica et al., 2014, Debruyne et al., 2008, Vandamme and De Ley, 1991). *Campylobacter* species have unique phenotypic traits. Under the microscope they appear as spirally curved-rods or S-shaped, 0.2 – 0.9 µm wide and 0.5 – 5 µm long. *Campylobacter* species are gram-negative, non-spore forming microorganisms (Vandamme and De Ley, 1991). Most *Campylobacter* species have a cock-screw like

motion with one or two flagella at one or both ends of the cell. In the entire *Campylobacter* genus, only *Campylobacter gracillis* is not motile and *Campylobacter showae* has multiple flagella (Debruyne et al., 2008).

C. jejuni is the only *Campylobacter* species that can hydrolyse Hippurate. Most species can reduce nitrate, they can also reduce fumarate to succinate. All species have oxidase activity and cannot liquefy gelatine and are non-haemolytic. They use a variety of organic acids such as amino acids as their source of carbon (Vandamme and De Ley, 1991).

Although *Campylobacter* species can tolerate elevated temperatures, they are not considered to be true thermophilic species because they are unable to grow at temperatures from 55ºC and above. Their ability to withstand elevated temperatures could be a direct reflection of how they adapted to their natural habitat which is the intestine of birds. Because of this feature *Campylobacter* species can also be referred to as thermotolerant species (Levin, 2007).

Freezing and thawing reduces the growth of *Campylobacter* species. Pure cultures of *Campylobacter* species are normally inactivated by freezing at –15ºC, whereas if the temperature is kept at 4ºC there is neither an increase nor decrease in the number of *Campylobacter* species present. Also, freezing does not eliminate *Campylobacter* species from contaminated food (Stern and Kotula, 1982). Therefore, it has been suggested that temperature control can be used as a measure to reduce the risk of contamination by inhibiting bacterial growth (Stern and Kotula, 1982).

The optimum pH range for *Campylobacter* species is 6.5 – 7.5 and they are found in the gastrointestinal tract of most animals and humans. *Campylobacter* species are non-spore forming, fastidious bacteria. Therefore, during isolation they require selective growth media because they are unable to ferment carbohydrates. Another important feature of *Campylobacter* species is that they are “microaerophilic” with a respiratory type of metabolism that requires special atmospheric conditions with low oxygen tension (Levin, 2007).

Campylobacter species are grown on selective media which contain one or more oxygen scavengers such as blood, ferrous iron, pyruvate and other agents such as antibiotics which eliminates microorganisms that might compete with *Campylobacter*

species (Garénaux et al., 2008). *Campylobacter* species are fastidious, microaerophilic and thermophilic but like many pathogenic microorganisms, they can adapt to unfavourable growth conditions (Garénaux et al., 2008). Rollins and Colwell (1986) reported on the growth of *Campylobacter* species in atmospheric conditions with high oxygen tension and observed that *Campylobacter* species change from spirally curved rods to coccoidal forms. *Campylobacter* species become dormant and limit their metabolic reactions in order to survival and not grow which makes them viable but non-culturable (VBNC) (Rollins and Colwell, 1986).

2.3. Pathogenesis of *Campylobacter* species

The term pathogenesis is used to describe the biological mechanism involved in the development of a disease (Rao et al., 2001). Understanding pathogenesis is important so that strategies of prevention can be formulated, vaccines can be designed, and cures discovered. The pathogenesis of campylobacteriosis to date is not completely understood but there are published results which can be used to further study how *Campylobacter* species become infectious (Silva et al., 2011; Van Vliet and Ketley, 2001).

Some of the virulence factors associated with *Campylobacter* species are motility and chemotaxis, adhesion and invasion, toxins, heat shock response, the ability to withstand bile salts and high temperatures (Konkel et al., 2001; Silva et al., 2011). For colonization of the gastrointestinal tract, movement of *Campylobacter* species into the mucus layer is very essential and its ability to penetrate the mucus barrier. Motility of *Campylobacter* can be detected by the presence of the flagellum which is an unsheathed polymer (Silva et al., 2011).

In *C. coli,* the flagellum consists of two homologous flagellin proteins, which is coded for by *flaA* and *flaB* genes. The role of the *flaA* gene is essential for the ability of the microbe to invade epithelial cells. The evidence of this was observed when *flaA* mutated which led to a truncated flagella filament composed of *flaB* with severe reduction in motility (Ketley and Konkel, 2005). A mutation in the *flaB* gene had no impact on the overall function of the flagellum compared to the structurally normal flagellum (Ketley and Konkel, 2005).

Motility of the pathogen is very important for colonization of the small intestine and motility increases under highly viscous conditions. *Campylobacter* species have the ability to successfully colonise the small intestine and can survive in the presence of gastric acids and bile salts (Van Deun et al., 2007). However, without the flagella, colonization of the small intestine by *Campylobacter* species is impossible. If colonization of the small intestine and the colon is successful, it can result in enterocolitis in susceptible hosts, (Van Deun et al., 2007; Van Vliet and Ketley, 2001).

Cytolethal Distending Toxin (CDT) is widely recognized as associated with Gram-negative bacteria and it is the best characterised of the toxins produced by *Campylobacter* species. It is described as an important virulence factor of *Campylobacter* species (Lara-Tejero and Galán, 2001). CDT is composed of three subunits encoded for by cdtA, cdtB and cdtC genes. The CdtB subunit has DNase-I like activity. Translocation of CdtB to the nucleus induces genotoxic effects on the host DNA therefore, triggering DNA repair cascades that lead to cell cycle arrest and eventually cell death (Ketley and Konkel, 2005, Lara-Tejero and Galán, 2001, Silva et al., 2011).

It is the responsibility of the CdtA and CdtC to deliver the CdtB subunit to the target cell, but also the binding of the CDT holotoxin to the cell membrane (Lara-Tejero and Galán, 2001). The CDT does not operate in isolation, since for it to induce enterocolitis the protein coded by the *flaA* gene facilitates adhesion of the microbe to the cell and cell invasion which result in cellular injury leading to a reduced absorptive capacity of the intestine (Van Deun et al., 2007). Although several virulence factors have been identified in *Campylobacter* species, such as flagella, bacterial adherence factors, invasiveness and production of toxins (Zilbauer et al., 2008, Van Vliet and Ketley, 2001), more work needs to be done to understand the mechanisms used by *Campylobacter* species to cause disease.

Of all the reported cases of *Campylobacter* enteritis, 10 – 20% reflect prolonged diarrhoea. It is common for *Campylobacter* infections to be undetected because most individuals have asymptomatic infections, which is prevalent in developing countries than in industrialized countries (Kaakoush et al., 2015).

2.4 Transmission of *Campylobacter* species

Transmission of *Campylobacter* to humans involves ingestion of faecal contaminated water, food and animal products (Weis et al., 2016; Luangtongkum et al., 2009). Vertical transmission of *Campylobacter* species in avian species (transmission from hen to developing chick) has not yet been clearly illustrated. Most studies have only focused on the horizontal transmission of *Campylobacter* species among birds or to humans. There are numerous reservoirs which play an intricate role in the transmission of *Campylobacte*r species such as poultry, domesticated animals and wild animals (Weis et al., 2016; Konkel et al., 2001). The prevalence of *C. coli* and *C. jejuni* within broiler chickens is high because *Campylobacter* species prefer the gastrointestinal tract of avian species as their natural habitat. Broiler chickens shed *Campylobacter* species into the environment via faeces and this has led to the abundance of *Campylobacter* species on poultry farms whereby it can be isolated from the water and food sources provided for chickens, the soil and the dust (Kaakoush et al., 2015).

Contaminated drinking water has also been linked with several human campylobacteriosis cases, particularly in developing countries where people consume faecal contaminated water because of limited resources. One source of water can be used for different activities such as drinking for animals and humans, bathing and washing of clothes. The very same stream could have soil with fertilizers being washed into it when it is rainfall season. This increases the risk of infection in humans because *Campylobacter* species (Kapperud et al., 2003).

Person to person transmission of *Campylobacter* species is rare and it only accounts for 3% of campylobacteriosis outbreaks documented by the Health Protection Agency in the United Kingdom and only 4% of the outbreaks in New Zealand (Gilpin et al., 2013; Little et al., 2010). Unpasteurised milk is also a vehicle for transmitting *Campylobacter* and between 2007 – 2009 the number of outbreaks linked to the consumption of unpasteurised milk increased from 30 to 51 in the United States (Mungai et al., 2015).

2.5 Antibiotics used for campylobacteriosis treatment

The use of antibiotics in patients with campylobacteriosis is rare because it is a self-limiting disease and therefore antibiotics are only used in severe cases (Coker et al., 2002). It is advisable for an individual with this infection to replenish lost fluids and electrolytes because one of the symptoms of campylobacteriosis is diarrhoea (Shobo et al., 2016). Macrolides, amoxicillin, fluoroquinolones are antibiotics which are frequently used for the treatment of severe cases of campylobacteriosis in humans (Bester and Essack, 2012, Pezzotti et al., 2003) and tetracyclines are recommended as the alternative drug of choice when the drugs listed before are not effective (Ghunaim et al., 2015). However, in the poultry industry antibiotics are frequently used for growth promotion and for the control, prevention and treatment of diseases. The immense use of antibiotics in animal husbandry is a major contribution to the emergence of antibiotic resistant strains of *Campylobacter* species (Moore et al., 2006).

Campylobacter species, such as *C. jejuni* in chickens, can adapt to the constant exposure to of antibiotics provided through feed and water and thus become resistant to these antibiotics (Ghunaim et al., 2015). This has a long-term effect because humans infected with antibiotic resistant *C. jejuni* will be limited to the kinds of antibiotics that can be used for treatment (Coker et al., 2002, Moore et al., 2006). The abuse of antibiotics in animal husbandry has led to an increase in antibiotic resistance strains which that can affect humans. In the agricultural sector, antibiotics are used in livestock as growth promoters and prophylaxis (Moyane et al., 2013) and this is due to lack of education in farmers and lack of regulation and monitoring as to what happens to the drugs sold and how much of them are used and for what purpose.

For diagnostic purposes antibiotic susceptibility can be tested for in several ways based on the special growth requirements of that microorganism, either by using the Kirby Bauer disc diffusion method (Biemer, 1973) or the minimum inhibitory concentrations (MICs) (Wiegand et al., 2008). The recommended method for testing antibiotic susceptibility in *Campylobacte*r species are the MICs. Broth microdilution, agar dilution and Epsilometer (E-Test) tests are some of the MIC tests which can be done on *Campylobacter* species however, broth microdilution and agar dilution are the two tests recommended by the Clinical and Laboratory Standards Institute (CLSI).

On a molecular level, polymerase chain reaction (PCR) is used to detect genes specific for antibiotic resistance but this is not used for diagnostic purposes (Luangtongkum et al., 2007). Coker et al., (2002) investigated *Campylobacter* ciprofloxacin resistance by screening for a single point mutation in the *gyrA* and the *parC* genes and erythromycin resistance by detecting mutations on the V domain of the 23S rRNA gene at position 2074 and 2075. *Campylobacter* tetracycline resistance has been screened for by detecting the presence of the *tetO* gene (Coker et al., 2002, Silva et al., 2011). Phenotypic results of the MICs can be used as guidelines for which genes to target using PCR (Luangtongkum et al., 2007).

There is a report of an increase in *Campylobacter* resistance to the newer classes of antibiotics such as macrolides, fluoroquinolones and tetracycline, all of which are recommended for the first line of antimicrobial treatment of campylobacteriosis in South Africa humans (Shobo et al., 2016). The use of antibiotics in the poultry industry of most developing countries like South Africa is not regulated unlike in developed countries such as Denmark, Sweden, Netherlands and the United Kingdom (Moyane et al., 2013). Therefore, in countries such as South Africa there is an urgent need to institute regulations, enforcement and monitoring to reduce antimicrobial resistance.

2.6 Prevalence of *Campylobacter* species worldwide

2.6.1 Campylobacteriosis in developed countries

There is an increase in the global incidence of campylobacteriosis (Altekruse et al., 1999; Coker et al., 2002; Kaakoush et al., 2015). In countries like Kenya it was reported in 2005 – 2007 that between of the 5.0 – 6.0% reported cases of gastroenteritis resulted in campylobacteriosis. Kaakoush et al., (2015) showed that there were 2.4 million cases of reported campylobacteriosis. In Australia, and in European and North American countries majority of the patients who consulted a general practitioner, admitted in hospitals, and were hospitalized for prolonged periods, was due to campylobacteriosis (Adak et al., 2005). The WHO reported an increase in the number of campylobacteriosis cases worldwide, with cases of the disease being higher than the combined cases of salmonellosis and yersiniosis (Silva et al., 2011).

Campylobacteriosis is a public health concern and therefore more effort should be made to understand the disease and to create awareness. For that to be achieved there should be enough data either on the reported cases of campylobacteriosis to study the trends and the patients. To also study the reservoirs of *Campylobacter* species, particularly poultry meat because it is the leading source of contamination linked to campylobacteriosis.

Different studies reflect variability in the isolation of *Campylobacter* species in poultry meat, more especially *C. coli* and *C. jejuni* which are predominantly isolated from poultry and poultry products. The differences may be due to the sensitivity and/or specificity of the test method used, unique food practices, the differences in the rigor of biocontrol protocols and the accessibility. Another important factor is the availability of resources provided by the country for disease surveillance. Most developing countries do not have good surveillance protocols in place for diseases which are not listed as a potential threat to humans or livestock (Kaakoush et al., 2015).

Similarly, the differences in the number of reported cases can be due to a phenomenon called the population-level immunity which refers to the ability of a population to protect itself using host immune responses against an infection. This affects the epidemiology of campylobacteriosis (Havelaar et al., 2009). Population level immunity is mostly seen in areas where *Campylobacter* is endemic and thus the infection is mainly observed in immune naïve children, with decreasing incidence associated with increasing age. Once a child is exposed, immunity against the infection is acquired (Havelaar et al., 2009). From the literature there are more case studies of patients who are under the age of 5 years being affected by campylobacteriosis. This shows that campylobacteriosis affects individuals who are not immune to this disease and once immunity is gained it lasts even though we do not know for sure how long.

Campylobacteriosis was one of the neglected foodborne pathogens. It is now being recognized worldwide because the number of cases being reported are increasing, ranging between 5.0 – 14.0% of the reported cases of gastroenteritis (Kaakoush et al., 2015). Although *Campylobacter* enteritis is usually not fatal, it can be fatal in immunocompromised individuals (Adak et al., 2005). An individual with a strong immune system can recover from a *Campylobacter* infection within a few days and therefore cases like these can go unreported (Havelaar et al., 2009).

In Bangladesh the prevalence of campylobacteriosis cases detected in diarrheal stool samples from children under five years of age was 39.0% (Kosek et al., 2003). In another study from Tanzania the prevalence of campylobacteriosis detection in stool samples from asymptomatic children less than 18 months old was 4.0% (Lindblom et al., 1995). In Trinidad a study was conducted by Rodrigo et al., (2005a) to analyse pathogens in water which was used to rinse broiler chicken carcasses in small processing operations. The targeted pathogens were *Campylobacter, Salmonella, Staphylococcus* and *Escherichia coli* using standard bacteriological methods. The collected water samples had a high prevalence of *Campylobacter* species (79.4%) and the predominant isolated species was *C. jejuni* (51.6%) followed by *C. coli* (49.4%). These results were consistent with studies on the distribution of *Campylobacter* species isolated from poultry (Rodrigo et al., 2005a). Thomas et al., (2006) reported that *E. coli* was the most frequently isolated bacteria (74.0%), followed by *Salmonella* and *Campylobacter* species from rinse and wash water used for broiler carcasses in several countries in the West Indies (Thomas et al., 2006).

Rodrigo et al., (2005b) investigated the prevalence of *Campylobacter* species in chickens sold by pluck shops in order to understand how sanitation practices at sale outlets influence contamination in these types of establishments. Standard bacteriological methods were used to test two types of samples, cloacal and carcass swab. The overall prevalence of *Campylobacter* in these samples was 82.0%. Carcass swab had the highest prevalence (83.9%) in comparison to cloacal swab (80.2%). The differences between the length of time the chickens were kept in the shop and the location of the carcasses for sale were statistically significant. The prevalence of *Campylobacter* species show that the processing methods increase the risk of cross-contamination. Infected chickens are washed in the same stagnant water after being plucked and eviscerated as are chickens that are not infected (Rodrigo et al., 2005b).

2.6.2 Campylobacteriosis in South Africa

In South Africa there were two interesting studies which were conducted on the prevalence of *Campylobacter* species. Obi et al., (2002) tested diarrhoea samples of Human Immunodeficiency Virus (HIV) positive patients with chronic diarrhoea in Venda, Limpopo. Of all the tested diarrhoea samples, 20% were positive for *Campylobacter* species by PCR. This prevalence closely matched that of another

study conducted by (Mackenjee et al., 1984) which focused on diarrheal cases of children under the age of five years and found that 21% of the diarrheal stools were positive for *Campylobacter* species.

In the northern region of South Africa Shobo et al., (2016) reported on the prevalence of *Campylobacter* species in different populations. The study showed higher rates of *Campylobacter* species in the population 40 – 49 years of age and the Human Immunodeficiency Virus (HIV) positive patients were high (27.0%) (Shobo et al., 2016). The study showed that HIV infection could have an impact on the infection rate. These findings were consistent with studies done worldwide where the prevalence of *Campylobacter* species is high in children under five years of age because they have no immunity against *Campylobacter*. Lack of immunity against Campylobacter infection increases the risk of infection when exposed to contaminated food or water.

The prevalence of *Campylobacter* infections is also influenced by seasons. A study conducted by (Rao et al., 2001) showed that there was a higher prevalence of *Campylobacter* enteritis during the warmer months than in the colder months. Also, poor hygiene, sanitation and close-proximity of humans and animals in developing countries were other risk factors identified by Lindblom et al., (1995) which facilitate a recurrent transmission of enteric pathogens including *Campylobacter* species.

Furthermore, the few studies which were done in South Africa are on the prevalence of *C. coli* and *C. jejuni* in the retail poultry industry and not in the informal poultry market which poses a threat to the public health because of the lack of regulations. The number of human clinical cases show that there is cause for concern and that strategies are needed to inform the global community on campylobacteriosis (Reddy and Zishiri, 2017; (Reddy and Zishiri, 2018; Van Nierop et al., 2005; Shobo et al., 2016; Moyane et al., 2013).

Most of the data presented here is on *Campylobacter* species in clinical human cases and not *Campylobacter* infections in poultry. There are no reported cases of chickens killed by *C. coli* or *C. jejuni.* Chickens are the primary reservoir host for *C. coli* and *C. jejuni* and thus acting as a primary source for human exposure

2.7 Diagnostic Tools

A good diagnostic tool should be cost effective, highly specific, sensitive, and have a short turnaround time. Isolation of *Campylobacter* species is tedious and time consuming due to its special growth/nutritional requirements and this would be a limiting factor in cases where results are needed quickly to make a diagnosis that could help treat the patient or stop the spread of infection (Kim et al., 2016).

Traditional methods which are commonly used for the isolation of *Campylobacter* species are time consuming, requires substantial amounts of the sample and skilled personnel (Kim et al., 2016). Also, competition from other microorganisms which grow faster compared to *Campylobacter* species leads reduction in numbers of *Campylobacter* species which makes it undetectable when tested (Fontanot et al., 2014). Due to their complex nutritional requirements and sensitivity to oxygen radicals *Campylobacter* species are grown on selective media which promotes growth for *Campylobacter* species and suppresses growth of other microorganisms (Garénaux et al., 2008).

2.7.1 Standard Bacteriological Methods

Campylobacter species are fastidious microorganisms, they are unable to degrade carbohydrates, and therefore they have special growth requirements. The normal habitat of *Campylobacter* species is in the gut of warm-blooded animals such as birds, goats, sheep and pigs (Kim et al., 2016, Levin, 2007). Because of that, isolation of *Campylobacter* species is done mostly on stool samples.

During processing of stool samples contact time with normal or high oxygen atmospheric conditions may be longer and microaerophilic gas generating sachets which are used for incubation may decrease in concentration. This might be unfavourable for bacterial growth. With bacteria evolving daily due to pressures of the environment and their need to survive, the use of antibiotics in selective media might not always work for the detection of all *Campylobacter* species. Some species of *Campylobacter* might be inhibited by antibiotics in media (Corry et al., 1995).

Most methods of *Campylobacter* species isolation involve pre-enrichment in a liquid medium before plating on solid agar. This step of pre-enrichment allows for the

damaged cells to recover and grow (Corry et al., 1995). Pre-enrichment of the sample is good for recovery, but it increases the total turnaround time. Some of the most common broths used for the pre-enrichment of *Campylobacter* species are Bolton Broth (BB), *Campylobacter* Enrichment Broth (CEB) and Preston Broth (PB) (Baylis et al., 2000). Baylis et al. (2000) found all three broths (CEB, PB and BB) to be equally effective for the isolation of *Campylobacter* species.

In addition to the enrichment step to isolate *Campylobacter* species, base agars are also used either for the detection or the enumeration of *Campylobacter* species. These include Preston Agar (PA), Charcoal Cefoperazone Deoxychocolate Agar (CCDA) and Butzler Agar (BA) (Baylis et al., 2000). In another study conducted by Zanetti et al., (1996) these three agar bases were tested for their efficacy and were found to be equally effective. In a study done by (Mason et al., 1999) the temperature for incubation was gradually increased from 37^{o}C up to the final incubation/optimum temperature of 41.5^{o}C and this allowed for further recovery of damaged cells. It is important to recover damaged cells so that the microorganism can be detected. Light microscopy is commonly used to identify the morphology of *Campylobacter* species after the colonies have been Gram-stained. For confirmation, biochemical tests are used along with the tests for antibiotic sensitivity (Zanetti et al., 1996)

The Centre for Disease Control and Prevention (CDC) (Atlanta, USA) conducted a surveillance study and incubated samples for 48 hrs and 72 hrs. After 48 hrs 66% of the cultures were negative, but after 72 hrs only 33% of the cultures were negative. Traditional culturing methods need more time to get accurate results and decrease the possibility of false negatives (Baylis et al., 2000).

2.7.2 Molecular Detection

Identification of *Campylobacter* species using standard bacteriological methods is a time-consuming process because of the bacterium's fastidious nature (Kim et al., 2016) and cannot be cultured when they enter the VBNC state (Fontanot et al., 2014). To overcome the long turnaround time for detecting and identifying *Campylobacter species* different diagnostic tools in molecular biology, have been designed. Some of these tools have reduced the total turnaround time and others allow differentiation of the bacteria into species and biotypes.

PCR technique is a molecular screening tool that uses specific primers for detection and as a diagnostic tool to speciate microbes which are difficult to isolate using standard bacteriological methods (Cao et al., 2012). PCR uses designed primers to amplify a specific region of the DNA which is unique to the organism, and the amplified DNA is viewed using gel electrophoresis which separates the bands. A molecular marker is used as a reference to determine the expected band sizes. The assay is highly sensitive in detecting the presence of small numbers of bacterial DNA and the total turnaround time is shorter than culturing the microorganism (Cao et al., 2012).

Leblanc-Maridor et al. (2011) designed a Real Time PCR (RT-PCR) assay specifically for the identification and quantification of *C. coli* and *C. jejuni* directly from faeces, feed and environmental samples. An enrichment step is still required for this assay although the total turnaround time is shortened. The three types of samples used in this study were used to identify the risk factors for contamination and transmission on a poultry farm (Laprade et al., 2016, Leblanc-Maridor et al., 2011). With this assay there is no waiting period because as the amplification is in process the results are read immediately on a computer.

Campylobacter species found in chicken faeces showed the chicken to be the carrier of the pathogen because the natural habitat of *Campylobacter* is in the gut of warm-blooded animals such as birds. *Campylobacter* species were found in feed, and thus the feed was changed, and the chickens were also treated with antibiotics to eradicate the microorganism. Because *Campylobacter* species were detected in the environmental samples, the environment was disinfected to kill the pathogens. This decreased risk of transmission of *Campylobacter* species among birds and cross contamination between feed and the environment. In this study only 5 µl of the extracted DNA was needed for the RT-PCR and showed high sensitivity (Leblanc-Maridor et al., 2011). RT-PCR is not a gold standard for detecting *Campylobacter* species however, this tool allows for rapid detection in real time, which is very important in diagnostics and can be used during outbreaks. This rapid diagnostic tool can be used alongside the traditional culturing method for confirming results.

Besides RT-PCR, there is a multiplex PCR (mPCR) which is used to detect more than one set of primer in one run. A mPCR is a diagnostic tool that can be used for the detection, speciation and characterisation of *Campylobacter* species into virulence

and antibiotic resistance genes (Persson and Olsen, 2005; Laprade et al., 2016). For *Campylobacter* species detection, Persson and Olsen, (2005) designed a mPCR for the genus identification of *Campylobacter* species and the speciation into either *C. coli* or *C. jejuni*. The diagnostic tool reduces the turnaround time further by having multiple runs in one.

Bessède et al. (2011) compared five *Campylobacter* detection methods to the standard culture method. Of the five methods, two were PCR tools and three were immunoenzymatic assays. The standard culture methods were different in how they were processed. One was taken through the filtration system before culturing onto blood agar and the other was not filtered but directly plated onto media with antibiotics (Karmali agar) (Bessède et al., 2011). Bessède et al. (2011) found that there was an inverse relationship between the sensitivity of the above-mentioned methods and their predictive values. The immunoenzymatic assays were the most sensitive tests, followed by the PCR methods and the standard culturing methods were the least sensitive. However, the positive predictive values of the immunoenzymatic assays were the lowest and the standard culturing methods had the highest positive predictive values. Although these rapid tests are very sensitive with a short turnaround time, they are more likely to give a false positive.

2.7.3 Serology

Standard bacteriological methods are effective for the diagnosis of an acute *Campylobacter* infection because only viable bacterial cells can be cultured on media (Vinueza-Burgos et al., 2017). Complicated cases of campylobacteriosis (GBS, MFS and ReA) cannot be easily linked to the source of infection because viable *Campylobacter* species will not be present in the stool samples at the time when symptoms appear, and a diagnosis is attempted. Therefore, the only way to show the aetiology of the disease is through serology by detecting antibodies, if they are present in the blood, using specific antigens to show if the host was previously exposed to any pathogen (Ang et al., 2007).

Agglutination, bactericidal, immunofluorescent antibody (IFA), enzyme immunoassay (EIA) and the complement fixation (CF) assays (Janvier et al., 2000) are examples of serological assays that have been developed for quantification of antibodies against

C. coli and *C. jejuni*. The CF assay is the only commercially available assay. This assay has high sensitivity which increases the chance of detection. Apart from being a time-consuming assay, antigenic cross reactivities is also a limiting factor when it comes to closely related species (Colmegna et al., 2004), it cannot be used to differentiate between *C. coli* and *C. jejuni* (Ang et al., 2007, Janvier et al., 2000). Most serological assays for detecting antibody to *C. coli* and *C. jejuni* are not validated because there are no set standards for determining seropositivity and no standard antigens (Schmidt-Ott et al., 2005).

2.8. Research Problem

The consumption of poultry and poultry products in South Africa is a common practice. According to the Department of Agriculture, Forestry and Fisheries (DAFF) there is an increase in the consumption of broiler chickens over the last several years. The number of chickens consumed from 2012 to 2014 has doubled and in 2014 the total consumption of all meat types (poultry, beef, lamb) was up to 2.9 million and 60% of that was from poultry. The annual report from the South African Poultry Association (SAPA) showed that in 2016 imported poultry was up to 560 155 tonnes which was higher than the total number of imported poultry meat from 2015 by 81 708 tonnes (SAPA, 2016).

As mentioned previously, poultry costs much less than pork, beef and lamb (National Agricultural Marketing Council, July 2015) and based on economic reasons it is cheaper to choose poultry meat than other meat as a source of protein. The increase in poultry consumption and its affordability has encouraged a steady growth in the informal poultry market (NAMC, 2015).

In South Africa regulation is carried out in abattoirs and in the formal retail poultry markets, but not in the informal poultry markets, where the risks of contamination by microbial pathogens are probably higher. Although the live chickens that are brought to the informal poultry market come from the regulated areas of the poultry market, there is little or no information as to what happens during the slaughtering and processing of chickens at the informal markets which includes defeathering, rinsing and packaging. Poultry meat can serve as a vehicle for foodborne pathogens such as *Salmonella, E coli*, *Campylobacter* species and *Staphylococcus aureus* and this is a

public health concern because of the high contamination risk during processing of the chickens (Moyane et al., 2013).

2.9. Aim and Objectives

The aim of this study was to determine the prevalence, characteristics and risk factors of *Campylobacter* species contamination of chicken carcasses sold at informal poultry outlets in Gauteng province, South Africa. The specific objectives of the project were to:

1. determine the different major methods used from slaughter to sale of chickens in the informal market,
2. determine the risk factors for contamination of carcasses with pathogens at the sale outlets,
3. determine the prevalence of *Campylobacter* on chickens sold in the informal market,
4. characterise the isolates of *Campylobacter* species regarding their species
5. determine the resistance of *Campylobacter* species recovered from chickens in the informal markets to commonly used antimicrobial agents in the poultry industry in Gauteng province and
6. make recommendations, based on the data generated from the study, on possible intervention strategy to reduce or eliminate the *Campylobacter* contamination risk posed to consumers of chickens from the informal market

Chapter 3: Materials and Methods

3.1 Study area, sample size determination and collection

3.1.1 Study Area

The Gauteng Department of Agricultural and Rural Development (GDARD) provided the research team with a list and locations of existing informal chicken outlets in Gauteng province. A total of 47 outlets were randomly selected from six townships in Gauteng (Fig. 3.1). Pre-study assessment visits were made to obtain information on the throughput, processing practices, GPS locations and the number of workers at each facility. A total of 61 consenting owners and outlets were considered eligible for inclusion in the study (3.1.2 Sample size determination). The study was conducted over a period of 8 months, August 2017 and concluding February 2018.

Figure 3.1: Gauteng province map, showing six areas where chicken carcasses were purchased for this study (GoogleMaps, 2018).

3.1.2 Determination of the number of samples and collection of samples from each outlet

The sample size was initially calculated according to the equation below (Thrushfield, 2017).

$$n_0 = \frac{\{1.96^2 \times P_{exp} \times (1 - P_{exp})\}}{d^2}$$

Where P_{exp} is the expected prevalence and d is the desired precision,

A P_{exp} value of 50% and a d value of 8% resulted in an estimated minimum sample size,

$$n_0 = \frac{\{3.84 \times 0.5 \times (0.5)\}}{0.0064} = 150$$

Therefore, the minimum sample size for whole chickens was determined to be 150. For this study, a total of 151 whole chicken carcasses were collected from 47 informal market outlets sampled (Appendix 5).

A total of 47 outlets were randomly selected for this study which are located within six townships in Gauteng. The number of samples from each outlet was determined according to the throughput assessed by the number of buckets or drums used for processing at each outlet. The outlets were selected based on four processing categories. In the first category, the outlets had over five drums of water used for rinsing chicken carcasses and over five people working in that operation. In the second category, the outlets had between two to five drums, which were used for rinsing water carcasses and two to five people involved in the operation. In the third category, the outlets had one individual running the entire operation using one bucket for rinsing. In the fourth category, the outlets had mechanical defeathering facilities with two to five people working in the operation.

Finally, the total number of carcass samples to be collected was determined by simple random sampling to achieve the sample size for the study. Therefore, the distribution of the samples collected from the six identified townships was as follows, 18 chicken carcasses were purchased from Garankuwa, 20 from Atteridgeville/Phomolong, 10 from Tembisa/Modise, 20 from Germiston, 20 from Alexandra and 60 from Soweto (Fig 3.1).

3.2 Questionnaire

Each selected outlet was visited for sampling during the period when active slaughtering and processing were taking place. During each visit, a pre-tested, standard questionnaire was administered to each outlet owner. The questionnaire captured the following information: the location of the outlet using GPS coordinates, chicken (sources, types and numbers of each type slaughtered daily), number of processing days per week, number of people involved in the operation, key steps in the operation, average number of hours or days birds are kept prior to slaughter, average weekly loss, due to death, of chickens pre-slaughter, average price of whole chicken, methods of disposal of solid and liquid waste from outlet and availability of refrigeration or freezing facilities on site (Appendix 1).

3.3 Sample processing and transportation to the laboratory

The purchased chickens were defeathered and eviscerated at each outlet. Whole dressed chicken carcasses were transported on ice in separate heavy-duty plastic bags and processed within 2 h in the post-mortem laboratory at the Department of Production Animal Studies, University of Pretoria, Faculty of Veterinary Science. The carcass drip from the heavy-duty plastic was collected in a sterile bottle (≈ 100 ml). The first swab was inserted into the cloaca of the carcass. Using a pair of scissors, the chicken carcasses were cut open and the second swab was rubbed on the outside surface and the internal parts of the open chicken carcass.

In this study the swabs taken from the cloacae were referred to as the cloacal swabs, the swabs applied to both the external and internal surfaces of the carcasses were referred to as carcass swabs. The fluids from the heavy-duty plastic bags collected in a sterile bottle were referred to as the carcass drip.

The two swabs (cloaca and carcass) were dipped separately in 5 ml of Amies Transport Medium (ATM) for further analysis. Carcass drip was collected in sterile screw-cap bottles to avoid spillage and contamination. All the samples were transported on ice and processed within 2 h of collection in the Bacteriology Department at OVR-ARC.

3.4 Laboratory procedures

3.4.1 Phenotypic identification of *Campylobacter* species

Isolation of *Campylobacter* species was conducted using standard bacteriological methods (Lior, 1984). The two swab samples (cloacal and carcass) were each added to 10 ml of Bolton Broth (BB) and incubated at 42^{o}C for 48 h. A 3 ml aliquot of carcass drip was centrifuged for 10 min at 425 *x g* (Eppendorf, India Ltd). The supernatant was discarded, and the pellet was re-suspended with 3 ml of buffered peptone water (BPW). One millilitre of the suspension was inoculated into 10 ml of BB and incubated at 42°C for 48 h after which 10 µl of the inoculated BB was plated onto *Campylobacter* blood-free agar containing Charcoal Cefoperazone Deoxycholate Agar (CCDA) selective supplement (Oxoid Ltd., Basingstoke, Hampshire, England). Inoculated BB was transferred into 1.5 ml Eppendorf tubes and stored at -80^{o}C until needed for further characterisation. The plates were incubated at 42°C for 48 h in a microaerophilic atmosphere containing 6% O_2 (10% CO_2 and 84% N_2) created by CampyGen sachet (Thermofischer Scientific, South Africa Pty Ltd) in an anaerobic jar (Thermofischer). Representative colonies on the blood-free *Campylobacter* agar, which were greyish with a running and non-translucent appearance were further analysed using the Gram stain reaction to determine if they were Gram-negative or Gram–positive and tested for catalase activity and oxidase hydrolysis. A positive catalase test was observed by bubbles when the catalase reagent, 3% hydrogen peroxide (Thermofischer Scientific, South Africa Pty Ltd) was added to the culture on a slide. A positive oxidase test was observed when a colony on a filter paper turned pink after the Kovacs Oxidase reagent (Thermofischer Scientific) was added directly onto the colony. Suspect colonies with a typical morphology following microscopic examination (Gram-negative, spirally curved rods), catalase-positive and oxidase-negative were classified as presumptive *Campylobacter* species. Presumptive *Campylobacter* isolates were sub-cultured onto blood agar and the plates were incubated as previously described. The resultant pure cultures were subjected to biochemical analysis focusing on DNase, Triple Sugar Iron (TSI) and Nitrate hydrolysis, identification as described by (Lior, 1984) and stored at - 80^{o}C for further analysis. *Campylobacter jejuni* (ATCC 33560) (Thermofischer) and *Campylobacter coli* (ATCC 43478) (Thermofischer) type strains were used as positive controls.

3.4.2 DNA extraction from cultures

DNA was extracted from pure cultures using heat method by boiling the bacterial suspension. The bacterial suspension (0.5 ml) was centrifuged for 5 min at 20,817 *x g* (Eppendorf, India Ltd). The supernatant was discarded, and the pellet was re-suspended in 200 µl of phosphate buffered saline (PBS) solution. The cell suspension was incubated using a heating block at 96^{o}C for 10 min. This was followed by placing the tubes containing the mixture directly on to ice (ice mixed with water) for cooling. The mixture was separated by centrifugation at 140 *x g* for 1 min and the supernatant was transferred into a clean labelled tube and stored at - 20^{o}C for further analysis.

3.4.3 Determination of DNA concentration

DNA concentration of the total genomic DNA extracted by the heat method was determined by using Qubit^{TM} dsDNA BR assay kit (Thermofischer Scientific, South Africa Pty Ltd) and all steps were performed according to the manufacturer's instructions.

3.4.4 Confirmation of *Campylobacter* isolates by PCR

Phenotypically identified isolates of *Campylobacter* species were confirmed by using conventional multiplex PCR (mPCR) assay (Persson and Olsen, 2005), with a slight modification regarding the amount of primers added to the reaction mixture. One of the genes (16S) was for genus-specific identification while the other two were for species differentiation, namely *C. coli* (Asp) and *C. jejuni (hipO)* (Whitehead Scientific, South Africa). The sequences and amplicon sizes are listed in Table 3.1. The reaction mixture contained 12.5 µl of Phusion Flash master mix (Thermofischer Scientific, South Africa Pty Ltd), 1 µl of each primer with a concentration of 20 µM, 1 µl of the DNA template and the total volume of 20 µl, was prepared with nuclease free distilled water.

Table 3.1: Primers used for genus identification and speciation of *Campylobacter* species into *C. coli* and *C. jejuni*

Gene target	Primers	Nucleotide sequence (5'-3')	Amplicon size (bp)	References
16S-rDNA	16S-F	-GGG AGG CAG CAG TAG GGA ATA-	1062	(Persson and
	16S-R	-TGA CGG GCG GTG AGT ACA AG-		Olsen, 2005)
Hipurrate	hipO-F	-GAC TTC GTG CAG ATA TGG ATG CTT-	344	(Persson and
Hydrolysis	hipO-R	-GCT ATA ACT ATC CGA AGA AGC CAT CA-		Olsen, 2005)
gene(*hipO*)				
Aspartokinase	CC118F	-GGT ATG ATT TCT ACA AAG CGA G-	500	(Linton et al.,
gene (*asp*)	CC519R	-ATA AAA GAC TAT CGT CGC GTG-		1997)

The PCR was conducted in a 96-well Thermal Cycler (Veriti, Applied Biosystems) with the following programme: one cycle of denaturation for 10 s at 98ºC, 30 cycles of annealing for 5 s at 57ºC and one cycle of extension for 20 s at 72ºC. Amplicons (5 µl) were separated on a 2% agarose gel stained with ethidium bromide. GeneRuler 1 kb DNA ladder (Thermofischer Scientific, South Africa Pty Ltd) was used as a molecular marker. *C. jejuni* (ATCC 33560) and *C. coli* (ATCC 43478) type strains were used as positive controls. The separated bands were viewed with a UV transilluminator (ChemiDoc™ XRS+, BioRad).

3.4.5 Antibiotic Susceptibility testing

To test for antimicrobial agent susceptibility, positive isolates stored at – 80ºC in 50% brain heart infusion (BHI)/50% glycerol were retrieved and inoculated in 10 ml of BHI. The tubes were incubated under microaerophilic conditions (6% O_2, 10% CO_2 and 84% N_2) created by a CampyGen sachet (Thermofischer Scientific, South Africa Pty Ltd) placed in a Oxoid™ Anaerobe 2.5L jar (Thermofischer Scientific, South Africa Pty Ltd) for 72 h at 42ºC. This was followed by plating 10 µl of the suspension onto blood agar and incubated as described above, after which the colonies were analysed by Gram staining, oxidase and catalase tests.

The above-mentioned procedure was repeated, and microaerophilic conditions were met by using a gas tank instead of a CampyGen sachet (Thermofischer Scientific, South Africa Pty Ltd). Colonies were analysed by Gram staining for the morphology and the primary biochemical tests were analysed by the oxidase and catalase tests.

3.4.6 Molecular characterisation of *Campylobacter* species by conventional PCR

3.4.6.1 DNA extraction from inoculated Bolton Broth (BB)

DNA was extracted from inoculated BB using heat by boiling. BB (0.5 ml) was incubated using the heating block at 96ºC for 10 min and the tubes were then cooled down at room temperature. The mixture was separated by centrifugation at 20 817 *x g* for 5 min and the supernatant was transferred into a clean labelled tube and stored at -20ºC for further analysis.

3.4.6.2 Determination of DNA concentration

Total genomic DNA concentration was determined according to the method as described in 3.4.3

3.4.6.3 Identification of *Campylobacter* species from inoculated BB by conventional PCR

Identification of *Campylobacter* species and speciation into *C. coli* and *C. jejuni* was determined according to the method described in 3.4.4

3.4.6.4 Characterisation of *C. coli* and *C. jejuni* using duplex and multiplex assays

Confirmed *C. coli* and *C. jejuni* isolates from the BB cultures were tested for the presence of six virulence genes (*dnaj, racR, ciaB, pldA, flaA, flaB),* three toxin releasing genes *(cdtA, cdtB, cdtC)* and one antibiotic resistance gene (*tetO*). The primers were obtained from Inqaba Biotech, South Africa. The method used to screen for these ten genes was adapted from a study conducted by (Laprade et al., 2016) with slight modifications in the amounts of primers added into the reaction mixture and the reduction in the number of cycles (Table 3.2). The reaction mixture contained 12.5 µl of Qiagen mPCR master mix kit (Whitehead Scientific, South Africa), and the primer concentration of 20 µM (0.25 – 1 µl) (Table 3.2), 2 µl of the template DNA and the mixture was made up to 25 µl with nuclease free water.

The PCR was conducted in a 96-well Thermal Cycler (Veriti, Applied Biosystems). The programme set to run the four assays had unique denaturation, annealing and initial elongation cycling conditions (Table 3.2). However, all four assays had an initial denaturation cycle set at 95^{o}C for 15 min and a final elongation cycle set at 72^{o}C for 10 min. Amplicons (5 µl) were separated on a 2% agarose gel stained with ethidium bromide. The separated bands were viewed with a UV transilluminator (ChemiDoc^{TM} XRS+, BioRad) and 1 kb DNA ladder (Thermofischer Scientific, South Africa Pty Ltd). *C. jejuni* (ATCC 33560) and *C. coli* (ATCC 43478) type strains were used as positive controls.

Table 3.2: PCR conditions, primer sequences and reaction volumes for the detection of six virulence genes, three toxin genes and one antibiotic resistance gene in *C. coli*- and *C. jejuni*-positive broth cultures

Gene target cycling conditions	Primers Reaction volume	Nucleotide Sequence (5'-3')	Amplicon size (bp)	References
Dnaj/racR/cdtC	dnajF	-AAG GCT TTG GCT CA-	720	(Datta et al.,
	dnajR	-CTT TTT GTT CAT CGT T-		2003)
Assay 1	**0.5 µl**			
94ºC for 1 min	racRF	-GAT GAT CCT GAC TTT G-	584	(Datta et al.,
46ºC for 1 min	racRR	-TCT CCT ATT TTT ACC C-		2003)
72ºC for 1 min	**1 µl**			
30 cycles	cdtCF	-TTG GCA TTA TAG AAA ATA CAG TT-	182	(Datta et al., 2003)
	cdtCR	-CGA TGA GTT AAA ACA AAA AGA TA-		
	0.25 µl			
tetO/cdtA	tetOF	-GGC GTT TTG TTT ATG TGC G-	559	(Kim et al., 2010)
	tetOR	-ATG GAC AAC CCG ACA GAA GC-		
Assay 2				
94ºC for 30 s	**0.25 µl**			
50ºC for 30 s	cdtAF	-CCT TGT GAT GCA AGC AAT C-	370	(Hickey et al.,
72ºC for 1 min	cdtAR	-ACA CTC CAT TTG CTT TCT G-		2000)
30 cycles				
	1 µl			
cdtB/ciaB/pldA	cdtBF	-CAG AAA GCA AAT GGA GTG TT-	620	(Datta et al.,
	cdtBR	-AGC TAA AAG CGG TGG AGT AT-		2003)
Assay 3	**1 µl**			
94ºC for 1 min				
58ºC for 1 min	ciaBF	-TGC GAG ATT TTT CGA GAA TG -	527	(Laprade et al.,
72ºC for 1 min	ciaBR	-TGC CCG CCT TAG AAC TTA CA-		2016)
30 cycles	**0.5 µl**			
	pldAF	-AAG AGT GAG GCG AAA TTC CA-	385	(Laprade et al.,
	pldAR	-GCA AGA TGG CAG GAT TAT CA-		2016)
	0.25 µl			
flaA/flaB	flaAF	-GCA AGA TGG CAG GAT TAT CA-	855	(Datta et al.,
	flaAR	-AAT AAA AAT GCT GAT AAA ACA G GT G-		2003)
Assay 4	**1 µl**			
94ºC for 1 min				
53ºC for 1 min	flaBF	-TAC CGA ACC AAT GTC TGC TCT GAT T-	260	(Goon et al.,
72ºC for 1 min	flaBR	-AAG GAT TTA AAA TGG GTT TTA GAA TAA ACA CC-		2003)
30 cycles				
	0.25 µl			

3.5 Data Analysis

The data obtained from the questionnaires were analysed using IBM SPSS statistics (Version 25). Collected data from the laboratory assays was captured on Excel (2016 version). Data was exported onto IBM SPSS statistics (Version 25) to calculate the frequency of detection of *Campylobacter* species (0 = absent, 1 = present). The relationship between the nine virulence genes and one antibiotic resistance gene detected in *C. coli* and *C. jejuni* were analysed using Stata™, Pearson correction, chi-square and logistic regression.

3.6 Approval of the study by the Ethics Committee

Approval was granted by the Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, Onderstepoort and the certificate number is v047-17 (Appendix 2). The Department of Agriculture Forestry and Fisheries (DAFF) approved storage of isolates from chicken carcasses at the Department of Veterinary Tropical Diseases (DVTD) and the Section 20 reference number is 12/11/1/1/8 (Appendix 3).

Chapter 4: Results

4.1 Location of identified informal markets

A total of 47 outlets of the informal market were visited over a period of 8 months. The outlets in six townships were either near the public transport stands (train station or taxi rank), in a house or by the side of a busy road. Majority (19.9%) of the informal outlets were located either close to the train station or a taxi rank. However, in areas such as Alexandra township, there were no street vendors selling slaughtered or live chickens. Individuals sold slaughtered chickens from their home and these home vendors were well known in the area.

4.2 Questionnaire

4.2.1 Processing methods

Three out of six of the selected townships in Gauteng slaughtered broiler chickens daily, with Alexandra and Tembisa/Modise slaughtering the most chickens at 70.0%, followed by Soweto with a low 8.3% (Table 4.1). Culled breeders were slaughtered daily in three areas. The highest daily number of culled breeders slaughtered were observed in Garankuwa (88.9%), followed by Germiston (60.0%) and the least number of culled breeders were slaughtered in Soweto (36.7%). Spent hens were slaughtered daily in all the six areas in different proportions. The outlets in Atteridgeville/Phomolong only slaughtered Spent hens (100.0%), followed by Soweto (55.0%), Germiston (40.0%), Tembisa/Modise (30.0%), Alexandra (30.0%) and Garankuwa (11.0%) ($P > 0.05$).

Only 75.7% of the chickens sold in the selected areas were kept in cages at the outlets. All the outlets (100.0%) in Germiston and Garankuwa kept the chickens on sale in cages. Alexandra had 70.0% of the chickens to be sold kept in cages, followed by Atteridgeville/Phomolong with 69.6%, Soweto with 65.0% and lastly Tembisa/Modise with 50.0%. In all six areas 100.0% of the chickens were slaughtered by knife and scalded. Evisceration of chickens varied from each area. Garankuwa, Germiston, Tembisa/Modise and Soweto outlets eviscerated all the chickens that were on sale. In Atteridgeville/Phomolong only 56.5% of the outlets eviscerated the chickens which

were on sale and outlets in Alexandra did not (0.0%) eviscerate chickens. (P > 0.05) (Table 4.1).

The six areas had different types of de-feathering methods. Outlets in Alexandra, Garankuwa and Germiston only used the hand-picking method (100.0%) to defeather the chickens which were on sale. The other three areas used a combination of two defeathering methods (hand-picking and knife shaving) *(*P > 0.05) (Table 4.1*).* In Atteridgeville/Phomolong, 69.6% and 30.4% of the outlets used the hand-picking and knife-shaving method respectively. In Soweto, 80.0% of the outlets used the hand-picking method and 20.0% used the knife shaving method. Outlets in Tembisa/Modise also made use of a combination of two defeathering methods, having most of the outlets using the knife shaving (70.0%) and 30.0% using the hand-picking method (P > 0.05*)* (Table 4.1*).* Outlets in Atteridgeville/Phomolong, Garankuwa and Tembisa/Modise used stagnant water in drums for rinsing chicken carcasses post-processing. Outlets in Alexandra and Germiston only used water in the bucket to rinse their chicken carcasses (100.0%). Outlets in Soweto had 51.7% using the bucket and 48.3% using stagnant water (Table 4.1).

From all the selected outlets, 49.0% of the chickens slaughtered daily were spent hens, 100.0% of all the outlets used knives to slaughter the chickens and 82.0% used the hand-picking method to defeather the chickens. From all the outlets, their source of water was either stagnant water (52.0%) or the bucket (47.0%). The processing methods which were investigated, were not statistically significant (P > 0.05) (Table 4.1) however the data collected gives an insight into the processing methods used in the Gauteng informal poultry market. Furthermore, the odds ratio showed that there could be an increase in the risk of *Campylobacter* contamination associated with slaughtering culled breeders (9.6), spent hens (7.2) and defeathering of chickens (1.7) (Table 4.3).

Table 4.1: Summary of processing methods used in the informal poultry outlets in six Gauteng townships

Parameter	No. (%) of outlets with practice in:											
	Alexandra*		Atteridgeville/ Phomolong*		Garankuwa*		Germiston*		Soweto**		Tembisa/Modise	
	No. (%) Positive	P-value	No. (%) Positive	P-value	No. (%) Positive	P-value	No. (%) Positive	P-value	No. (%) Positive	P-value	No. (%) Positive	P-value
Types of birds slaughtered daily												
Broilers	14 (70.0)	0.2419	0 (0.0)	0.4226	0 (0.0)	0.3555	0 (0.0)	0.1994	5 (8.3)	0.1334	7 (70.0)	0.2419
Culled breeders	0 (0.0)		0 (0.0)		16 (88.9)		12 (60.0)		22 (36.7)		0 (0.0)	
Spent hens	6 (30.0)		23 (100.0)		2 (11.1)		8 (40.0)		33 (55.0)		3 (30.0)	
Chickens kept in cages												
Yes	14 (70.0)	0.2422	16 (69.6)	0.2375	18 (100.0)	0.5000	20 (100.0)	0.5000	39 (65.0)	0.1855	5 (50.0)	a*
No	6 (30.0)		7 (30.4)		0 (0.0)		0 (0.0)		21 (35.0)		5 (50.0)	
Chickens slaughtered with knives												
Yes	20 (100.0)	0.5000	23 (100.0)	0.5000	18 (100.0)	0.5000	20 (100.0)	0.5000	60 (100.0)	0.5000	10 (100.0)	0.5000
No	0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)	
Scalding												
Yes	20 (100.0)	0.5000	23 (100.0)	0.5000	18 (100.0)	0.5000	20 (100.0)	0.5000	60 (100.0)	0.5000	10 (100.0)	0.5000
No	0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)	
Eviscerated chickens												
Yes	0 (0.0)	0.5000	13 (56.5)	0.0826	18 (100.0)	0.5000	20 (100.0)	0.5000	14 (23.3)	0.3119	10 (100.0)	0.5000
No	20 (100.0)		10 (43.5)		0 (0.0)		0 (0.0)		46 (76.7)		0 (0.0)	
Type of defeathering method												
Hand-picking	20 (100.0)	0.5000	16 (69.6)	0.2375	18 (100.0)	0.5000	20 (100.0)	0.5000	48 (80.0)	0.3440	3 (30.0)	0.2422
Knife shaving	0 (0.0)		7 (30.4)		0 (0.0)		0 (0.0)		12 (20.0)		7 (70.0)	
Type of rinsing method												
Bucket	20 (100.0)	0.5000	0 (0.0)	0.5000	0 (0.0)	0.5000	20 (100.0)	0.5000	31 (51.7)	0.0212	0 (0.0)	0.5000
Stagnant water in drums	0 (0.0)		23 (100.0)		18 (100.0)		0 (0.0)		29 (48.3)		10 (100.0)	

Based on the total number of samples collected from each township: Alexander (20 samples), Atteridgeville/Phomolong (23 samples), Garankuwa (18 samples), Germiston (20 samples), Soweto (60 samples), Tembisa/Modise (10 samples). [a = No statistics could be computed because the parameter is constant]

4.2.2 Risk factors for contamination of carcasses with *Campylobacter* species

Risk factors for contamination of chicken carcasses across 47 informal outlets were evaluated with consideration to four aspects. The location (bucket /counter /drums /freezer) of the chicken carcasses for sale, the length of time it is displayed on the counter at ambient temperatures, if the knife used for slaughtering the chicken was washed or not and lastly the observed level of sanitation of the entire operation including the person processing the chickens (Appendix 4).

Overall, a majority of the outlets in Gauteng placed their slaughtered chickens in the bucket (42.0%) and some displayed them on the counter (35.0%). Only a few outlets placed their slaughtered chickens in the freezer (6.2%) and it was quite rare to find those that placed them in the drums (3.0%). From the 47 outlets sampled only 46.0% washed their knives after slaughtering the chicken. Most of the outlets had poor sanitation (60.0%) (Table 4.2). The differences in the risk factors for carcass contamination were statistically significant ($P < 0.05$) for one parameter in Soweto, whereby owners not washing their knives after slaughtering each chicken increased the risk of *Campylobacter* contamination and the other five areas (Atteridgeville/Phomolong, Tembisa/Modise, Germiston, Garankuwa and Alexandra) had no significant findings in that parameter ($P > 0.05$) (Table 4.2).

Risk factors for contamination of carcasses at each outlet are associated with the practices or exposure. Table 4.3 lists the factors associated with contamination of *Campylobacter* and likelihood of occurrence (Odds ratio). Slaughtering culled breeders was a statistically significant higher risk factor associated with contamination (OR = 9.7) of *Campylobacter* species compared to slaughtering spent hens (OR = 7.2) and this is followed by poor sanitation (OR = 2.3). The use of stagnant rinsing water for slaughtered chickens (OR = 2.0) and defeathering were of statistically significantly lower risk of contamination (with OR = 1.787483) in comparison to the above-mentioned parameters ($P < 0.05$) Table (4.3).

Table 4.2: Summary of risk factors for carcass contamination with *Campylobacter* species in six Gauteng townships

Risk factor	No. (%) of outlets with practice in:											
	Alexandra		Atteridgeville/ Phomolong		Garankuwa		Germiston		Soweto		Tembisa/Modise	
	No. (%) Positive	p-value	No. (%) Positive	p-value	No. (%) Positive	p-value	No. (%) Positive	p-value	No. (%) Positive	p-value	No. (%) Positive	p-value
Location of the carcass for sale												
Buckets	6 (30.0)		10 (43.5)		0 (0.0)		0 (0.0)		43 (71.7)		5 (50.0)	
Counter	14 (70.0)	0.2288	7 (30.4	0.0712	18 (100.0)	0.3910	0 (0.0)	0.3910	9 (15.0)	0.2142	5 (50.0)	0.1817
Drums	0 (0.0)		6 (26.1)		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)	
Freezer	0 (0.0)		0 (0.0)		0 (0.0)		20 (100.0)		8 (13.3)		0 (0.0)	
Length of the carcass on the counter												
<30 min	8 (40.0)		N/A		18 (100.0)		0 (0.0)		21 (35.0)		0 (0.0)	
30-60 min	6 (30.0)	0.0632	N/A	N/A	0 (0.0)	0.3910	0 (0.0)	0.3910	15 (25.0)	0.0673	0 (0.0)	0.1817
>60 min	0 (0.0)		N/A		0 (0.0)		0 (0.0)		24 (40.0)		5 (50.0)	
N/A	6 (30.0)		N/A		0 (0.0)		20 (100.0)		0 (0.0)		5 (50.0)	
Owners wash knives after slaughtering												
No	6 (30.0)	0.2422	17 (73.9)	0.2840	18 (100.0)	0.5000	8 (40.0)	0.1257	32 (53.3)	0.0424	0 (0.0)	0.5000
Yes	14 (70.0)		6 (26.1)		0 (0.0)		12 (60.0)		28 (46.7)		10 (100.0)	
Overall level of sanitation												
Fair	6 (30.0)		2 (8.7)		0 (0.0)		20 (100.0)		3 (5.0)		0 (0.0)	
Good	13 (65.0)	0.1955	0 (0.0)	0.3705	0 (0.0)	0.4226	0 (0.0)	0.4226	5 (8.3)	0.3380	10 (100.0)	0.4226
Poor	1 (5.0)		21 (91.3)		18 (100.0)		0 (0.0)		52 (86.7)		0 (0.0)	

*Based on conventional and PCR methods

Table 4.3: Odds ratio for risk factors associated with *Campylobacter* species contamination at the outlets in six Gauteng townships

Risk Factor	***Odds Ratio***	***P- value***
Culled breeders	9.674699	0.000
Spent hens	7.248227	0.000
Defeathering	1.787483	0.041
Stagnant water	2.046823	0.006
Poor sanitation	2.583804	0.002

4.3 Phenotypic identification of *Campylobacter* species and speciation

4.3.1 Frequency of *Campylobacter* species

Overall, using phenotypic identification, the frequency of isolation of *Campylobacter* species was highest in carcass drip (40.4%) and lowest in carcass swab (24.5%) while 37.7% of cloacal swabs yielded the organism ($P < 0.05$) (Table 4.4). For carcass swab samples collected across the six sampling areas, the frequency of isolation of *Campylobacter* species ranged from 0.0% (Tembisa/Modise) to Garankuwa (50.0%) and the differences between the two areas were statistically significant ($P = 0.0091$) (Table 4.4).

For cloacal swab samples, the lowest frequency of isolation of *Campylobacter* species was found in Tembisa/Modise (0.0%) while the highest frequency was detected in Germiston (65.0%). The differences between the two areas were statistically significant ($P = 0.0235$). The frequency of isolation of *Campylobacter* species ranged from 0.0% (Tembisa/Modise) to 100.0% (Germiston) for carcass drip samples and the difference was statistically significant ($P= 0.0043$) (Table 4.4).

Within the six sampled areas, differences in the frequency of isolation of *Campylobacter* species among the three types of samples (carcass swab, cloacal swab and carcass drip) were statistically significantly in three areas which are Atteridgeville/Phomolong ($P = 0.0279$), Alexandra ($P = 0.0377$) and Soweto ($P = 0.0429$). In total for the three types of samples (453 samples) investigated across the six areas, 155 (34.2%) were positive for *Campylobacter* species. The frequency of isolation using standard bacteriological methods ranged from 0.0% (0/30) in

Tembisa/Modise to 66.7% (40/60) in Germiston. The differences by location were statistically significant (P = 0.0201) (Table 4.4).

Table 4.4: Frequency of *Campylobacter* species isolation from carcass drip, cloacal and carcass swab using standard bacteriology methods

Area	No. of Samples tested	No. (%) Positive* for *Campylobacter* species			p-value	Total for all Sources	
		Carcass swab	Cloacal swab	Carcass drip		No. Tested	No. (%) Positive
Atteridgeville/Phomolong	23	7 (30.4)	13 (56.5)	11 (47.8)	0.0279	69	31 (44.9)
Garankuwa	18	9 (50.0)	7 (38.9)	1 (5.5)	0.1425	56	17 (30.4)
Tembisa/Modise	10	0 (0.0)	0 (0.0)	0 (0.0)	a**	30	0 (0.0)
Alexandra	20	4 (20.0)	4 (20.0)	7 (35.0)	0.0377	60	15 (25.0)
Germiston	20	7 (35.0)	13 (65.0)	20 (100.0)	0.0710	60	40 (66.7)
Soweto	60	10 (16.7)	20 (33.3)	22 (36.6)	0.0429	180	52 (28.9)
p-value		0.0091	0.0235	0.0443			0.0201
Total	151	37 (24.5)	57 (37.7)	61 (40.4)	0.0077	453	155 (34.2)

*Based on isolation using standard bacteriological methods

a* = No statistics could be computed because the parameter is constant

4.3.2 Confirmation of *Campylobacter* isolates by conventional PCR

The frequency of detection by conventional PCR from recovered *Campylobacter* isolates is shown in Table 4.5. Of a total of 155 isolates phenotypically identified as *Campylobacter* species, 106 (68.4%) were confirmed by PCR. The frequency of PCR-confirmed *Campylobacter* species by location of the markets ranged from 6.7% (Alexandra) to 88.2% (Garankuwa) and the differences between the five areas were statistically significant (P = 0.0276) (Table 4.5).

For the five townships from which the isolates originated, the frequency of detection of *Campylobacter* species was 70.3% (26/37), 68.4% (39/57) and 67.2% (41/61) from carcass swabs, cloacal swabs and carcass drip respectively. The frequency of detection of *Campylobacter* species by PCR varied significantly across the three types of samples, for isolates recovered from carcass swab (P = 0.0310), cloacal swabs (P = 0.0287) and carcass drip (P = 0.0274) (Table 4.5).

For *Campylobacter* species recovered from the three types of samples within each geographic location, statistically significantly differences in frequency were detected in three of five areas, namely, Atteridgeville/Phomolong (P = 0.0142), Garankuwa (P = 0.0130) and Soweto (P = 0.0267) (Table 4.5). Isolates positive for the genus *Campylobacter* which were identified by conventional microbiological methods were

speciated into *C. coli* and *C. jejuni* using conventional PCR. Overall, out of the 106 (68.4%) conventional PCR confirmed isolates of the genus *Campylobacter,* 6.6% were confirmed *C. coli* and 6.6% *C. jejuni species.* The rest were neither *C. jejuni* nor *C. coli.*

Table 4.5: Confirmation of *Campylobacter* isolates detected by using conventional Polymerase Chain Reaction (PCR) method

Area	No. (%) Positive* for *Campylobacter* species						p-value	Total of all Sources	
	No. Tested	Carcass swab	No. Tested	Cloacal swab	No. Tested	Carcass drip		No. Tested	No. (%) Positive
Atteridgeville/Phomolong	7	7 (100.0)	13	6 (42.2)	11	9 (81.8)	0.0142	31	22 (70.9)
Garankuwa	9	8 (88.9)	7	6 (85.7)	1	1(100.0)	0.0130	17	15 (88.2)
Alexandra	4	0 (0.0)	4	1 (25.0)	7	0 (0.0)	0.4226	15	1 (6.7)
Germiston	7	3 (42.8)	13	12 (92.3)	20	17 (85.0)	0.1212	40	32 (80.0)
Soweto	10	8 (80.0)	20	14 (70.0)	22	14 (63.6)	0.0267	52	36 (69.2)
p-value		0.0310		0.0287		0.0274			0.0276
Total	37	26 (70.3)	57	39 (68.4)	61	41 (67.2)		155	106 (68.4)

*Based on detection using conventional PCR

4.4 Molecular characterisation of *Campylobacter* species by mPCR

4.4.1 Screening of inoculated Bolton broth (BB) by conventional PCR

The genus *Campylobacter* was detected by a 1062 bp band on 2% agarose gel (Fig. 4.1).

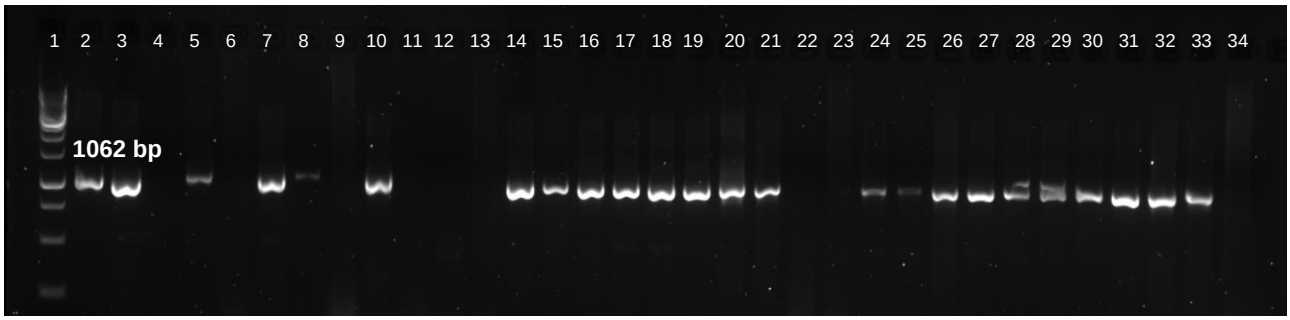

Figure 4.1: *Campylobacter* species 16s rDNA PCR results.
Lane 1 is the 1kb GeneRuler (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is the positive control, *C. jejuni* (ATCC 33560) and **Lane 3** is the second positive control, *C. coli* (ATCC 43478). **Lane 4** is the negative control and **Lanes 5-33** are the tested broth samples from Atteridgeville/Phomolong with the expected band size of 1062 bp.

Carcass swabs had the highest frequency of *Campylobacter* (99.2%), followed by cloacal swabs (98.4%) and the carcass drip (82.8%). The differences in prevalence among the three types of samples were statistically significant for carcass swab samples (P = 0.0452) and cloacal swab samples (P = 0.0468) (Table 4.6). The overall frequency of detection of *Campylobacter* species from these five areas was 93.5%.

The differences in the detection rate for *Campylobacter* species in the three types of samples within each of the areas were only statistically significantly different in Garankuwa (P = 0.0004), Tembisa/Modise (P = 0.0012) and Alexandra (P = 0.0009). Overall, of the 384 BB samples tested, 359 (93.5%) yielded *Campylobacter* species using conventional PCR. Soweto had the highest frequency of detection (100%), followed by Garankuwa (98.1%), Alexandra (95.0%), Tembisa/Modise (96.7%) and Germiston (66.7%). The difference in prevalence in the five areas was statistically significant (P < 0.05) within three areas (Garankuwa, Tembisa/Modise and Alexandra) as shown in (Table 4.6).

Table 4.6: Frequency of *Campylobacter* species detection from inoculated BB culture samples using conventional PCR method

Area	No. (%) Positive* for *Campylobacter* species				p-value	Total for all sources	
	No. tested	Carcass swab	Cloacal swab	Carcass drip		No. tested	No. (%) Positive
Garankuwa	18	18 (100.0)	18 (100.0)	17 (94.4)	0.0004	54	53 (98.1)
Tembisa/Modise	10	10 (100.0)	10 (100.0)	9 (90.0)	0.0012	30	29 (96.7)
Alexandra	20	19 (95.0)	18 (90.0)	20 (100.0)	0.0009	60	57 (95.0)
Germiston	20	20 (100.0)	20 (100.0)	0 (0.0)	0.1835	60	40 (66.7)
Soweto	60	60 (100.0)	60 (100.0)	60 (100.0)	a*	180	180 (100.0)
p-value		0.0452	0.0468	0.1087			0.0594
Total	128	127 (99.2)	126 (98.4)	106 (82.8)		384	359 (93.5)

*Based on detection using conventional PCR

a* = No statistics could be computed because the parameter is constant

4.4.2 Speciation of identified *Campylobacter* species into *C. coli* and *C. jejuni*

Speciation of *Campylobacter* species into *C. coli* and *C. jejuni* was done through detected by 500 bp (Fig 4.2) and 344 bp (Fig 4.3) bands respectively on 2% agarose gel.

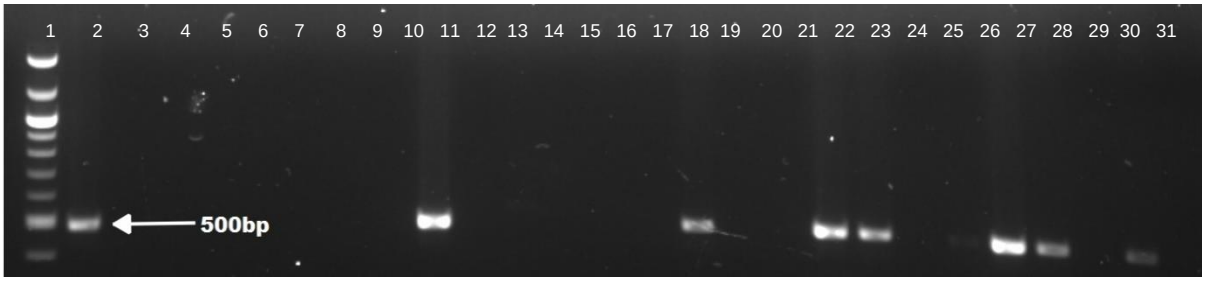

Figure 4.2: PCR amplicon results for speciation of *C. coli* using the Aspartokinase (Asp) primers. **Lane 1** is the 1 kb gene ruler (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is the positive control, *C. coli* (ATCC 43478) and **Lane 3** is the negative control. **Lane 4-31** are the inoculated broth culture samples from Tembisa/Modise which were positive for the genus *Campylobacter* species with the expected band size of 500 bp.

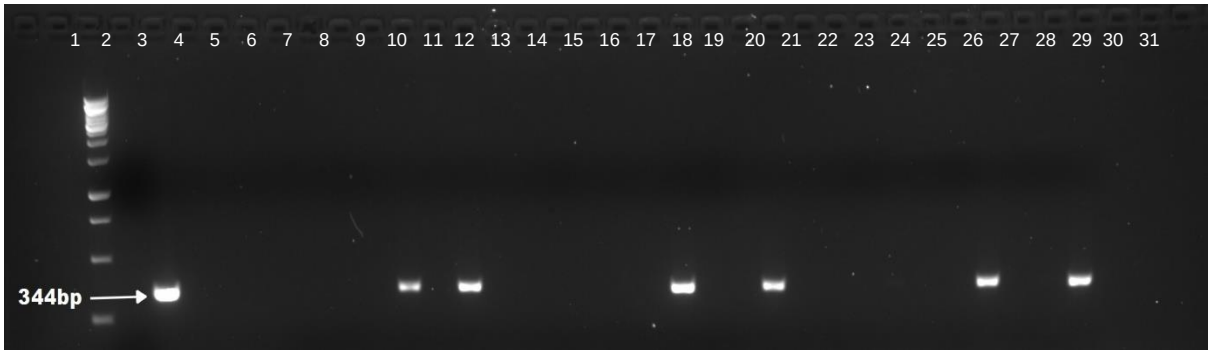

Figure 4.3: PCR amplicon results for speciation of *C. jejuni* using the Hippurate (hipO) primers. **Lane 1** is the 1 kb GeneRuler (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is empty, **Lane 3** is the positive control, *C. jejuni* (ATCC 33560) and **Lane 4** is the negative control. **Lanes 5-31** are the tested inoculated broth cultures from Tembisa/Modise which were positive for the genus *Campylobacter* species with the expected band size of 344 bp.

From the 384 BB samples tested, 340 (88.5%) were confirmed by PCR to be *Campylobacte*r species and of these, 54 (15.0%, P = 0.0108) and 27 (7.5%, P = 0.0952) were identified as *C. coli* and *C. jejuni* respectively (Table 4.7). For *C. coli* across the five areas, the frequency ranged from 7.0% (Alexandra) to 40.0% (Germiston) and the differences across the five areas were statistically significant (P = 0.0108*)* while for *C. jejuni*, the lowest frequency was 0.0% (Germiston and Soweto) and the highest frequency was 22.8% (Alexandra) and the difference were not statistically significant (P = 0.0952) (Table 4.7). The difference in the frequency of detection of *C. coli* and *C. jejuni* for samples collected from outlets was only statistically significantly different in Tembisa/Modise (P = 0.0489*),* with *C. coli* (24.1%) and *C. jejuni* (20.7%) (Table 4.7).

Table 4.7: Frequency of Speciation of *C. coli* or *C. jejuni* detected by using conventional PCR method

Area	No. tested	*C. coli*	*C. jejuni*	P-value
		No. (%) positive		
Garankuwa	53	11 (20.8)	8 (15.1)	0.0997
Tembisa/Modise	29	7 (24.1)	6 (20.7)	0.0489
Alexandra	57	4 (7.0)	13 (22.8)	0.3100
Germiston	40	16 (40.0)	0 (0.0)	0.5000
Soweto	180	16 (8.9)	0 (0.0)	0.5000
p-value		0.0108	0.0952	
Total	359	54 (15.0)	27 (7.5)	

4.4.3 Characterisation of *C. coli* and *C. jejuni* using duplex and multiplex PCR assays

The first assay was a mPCR which targeted *dnaj* (720 bp) virulence gene, *racR* (584 bp) virulence gene and *cdtC* (182 bp) toxin gene (Fig 4.4). The second assay was a duplex PCR which targeted *tetO* (559 bp) antibiotic resistance gene and *cdtA* (370 bp) toxin gene (Fig 4.5). The third assay was also a mPCR which targeted *cdtB* (620 bp) toxin gene, *ciaB* (527 bp) virulence gene and *pldA* (385 bp) virulence gene (Fig 4.6). The fourth assay was also a duplex assay which targeted *flaA* (855 bp) virulence gene and *flaB* (260 bp) virulence gene (Fig 4.7). The ten amplified genes were visualised on 2% agarose gel.

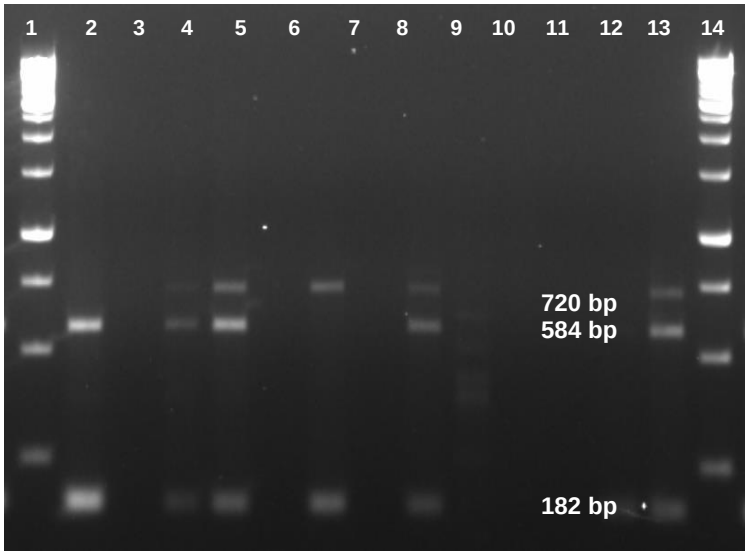

Figure 4.4: PCR amplicon results for Assay 1 (targeting *dnaj, racR* and *cdtC* genes). **Lane 1** is the 1kb gene ruler (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2-13** are the *C. jejuni*-positive broth cultures from Garankuwa which were tested for the three genes. The expected sizes were 720 bp for dnaj, 584 bp for racR and 182 bp for cdtC. **Lane 14** is the positive control, *C. jejuni* (ATCC 33560).

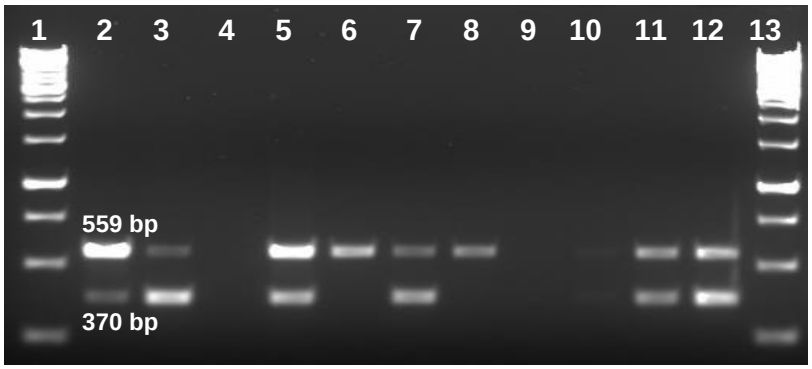

Figure 4.5: PCR amplicon results for Assay 2 (targeting *tetO* and *cdtA* genes). **Lane 1 & 13** are the 1kb gene rulers (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is positive control, *C. jejuni* (ATCC 33560). **Lane 3-12** are the *C. coli*-positive broth cultures from Tembisa which were tested for the two genes. The expected band sizes were 559 bp for *tetO* and 370 for *cdtA.*

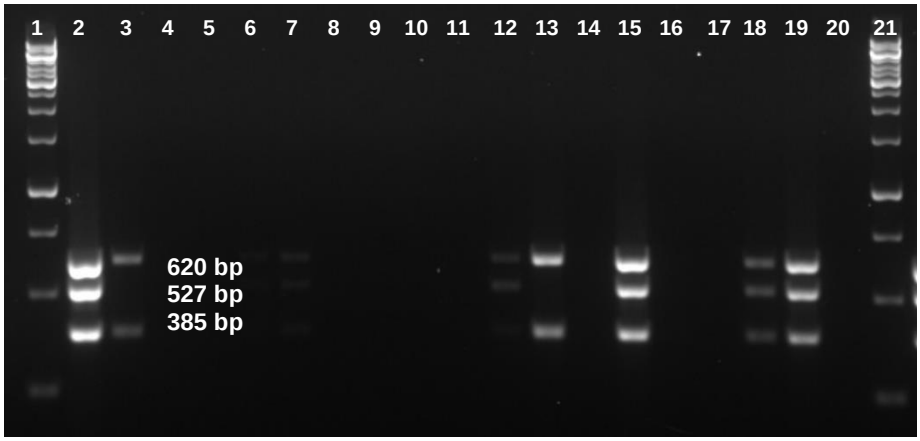

Figure 4.6: PCR amplicon results for Assay 3 (targeting *cdtB, ciaB* and *pldA* genes). **Lane 1 & 21** are the 1kb gene rulers (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is positive control, *C. jejuni* (ATCC 33560). **Lane 3-19** are the *C. coli* positive broth cultures from Alexandra which were tested for the three genes. The expected band sizes were 620 bp for *cdtB,* 527 bp *ciaB* and 385 bp for *pldA.* **Lane 20** is the negative control.

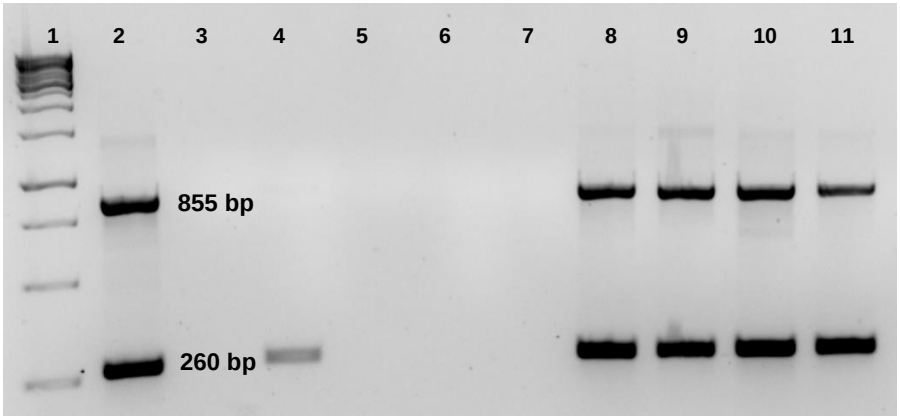

Figure 4.7: PCR amplicon results for Assay 4 (targeting *flaA* and *flaB* genes). **Lane 1** is the 1kb gene ruler (Thermofischer Scientific, South Africa Pty Ltd). **Lane 2** is positive control, *C. jejuni* (ATCC 33560). **Lane 3** is the negative control. **Lane 4-11** are the *C. jejuni*-positive broth cultures tested samples from Garankuwa with the expected bands of 855 bp for *flaA* and 260 bp for *flaB* PCR products.

The frequency of detection of the six virulence genes, three toxin genes and one resistance genes are summarised in Table 4.8 for *C. jejuni* positive broth cultures and Table 4.9 for *C. coli* positive broth cultures. Overall, the frequency of detection of virulence and toxin genes was higher in *C. jejuni* positive broth cultures than in *C. coli* broth cultures. The only exception was observed in *flaB* gene which was higher in *C. coli* broth cultures than in *C. jejuni* broth cultures (Fig. 4.8).

For the virulence genes, the frequency of detection was lowest for *Dnaj* (53.6%) and highest for *ciaB* and *pldA* (75.0%) while for toxin genes, the frequency ranged from 67.9% (*cdtA* and *cdtC*) to 78.6% *(cdtB).* Across the three areas, the frequency of detection of toxin genes was statistically significantly different for only *cdtA* gene (P = 0.0488).

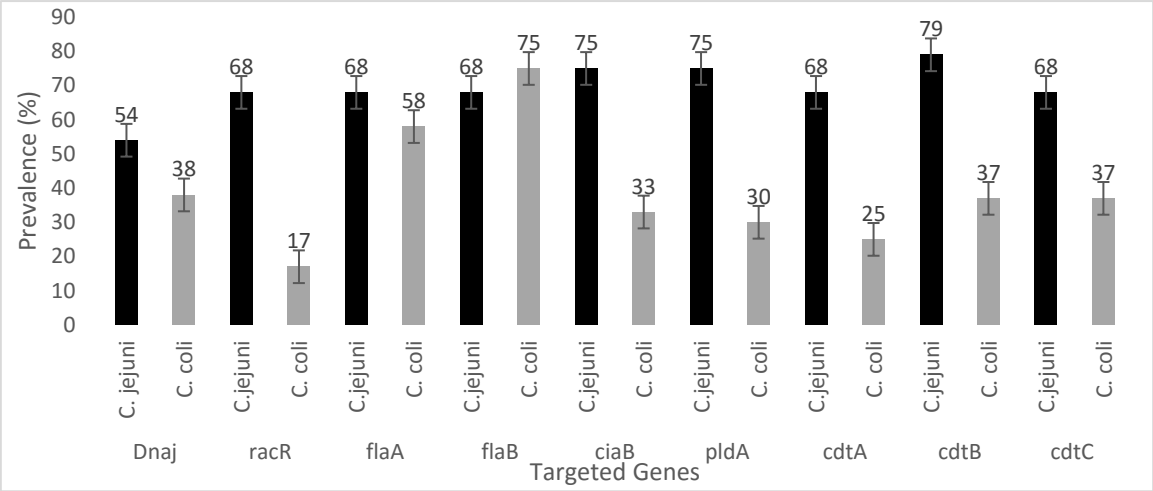

Figure 4.8: Frequency of detection of six virulence (*dnaj, racR, pldA, ciaB, flaA, flaB*) and three toxin genes (*cdtA, cdtB, cdtC)* in *C. jejuni*- and *C coli*-positive broth cultures.

Alexandra had the highest frequency of detection in the following targeted genes, *flaA/flaB/ciaB/pldA/cdtA* (84.8%), followed by *cdtC/racR* (76.9%) and *dnaj/cdtA* (69.2%). The differences in the frequency of detection of virulence and toxin genes in Alexandra was statistically significant (P < 0.05) (Table 4.8). Compared to Alexandra and Tembisa/Modise, Garankuwa had the lowest frequency of detection of virulence and toxin genes. The frequency of detection between virulence and toxin genes in Garankuwa and Tembisa/Modise was significantly different (P < 0.05) (Table 4.8).

The antibiotic resistance gene (*tetO*) was detected at the highest frequency in Tembisa/Modise (83.3%), followed by Garankuwa (44.4%) and the lowest was Alexandra (36.7%). The frequency of detection of the antibiotic resistance gene in *C. jejuni* broth cultures was not statistically significant (P > 0.05) (Table 4.8).

Positive *C. coli* broth cultures had a lower frequency of detection of virulence genes (38.7%) (Table 4.8) compared to *C. jejuni* positive samples (69.0%) (Table 3.9),

Germiston had the highest frequency of detection of virulence genes in *flaB* (95.2%), followed by *flaA* (71.4%), *ciaB*/*pldA*/*cdtB* (57.1%), *dnaj*/*cdtC* (47.6%), *cdtA* (33.3%) and *racR* 14.3%.

From the five areas targeted, Alexandra had the lowest frequency of isolation (Table 4.9). The difference in prevalence of virulence and toxin genes in the targeted areas was statistically significant ($P < 0.05$) (Table 4.9).

The antibiotic resistance gene *(tetO)* was detected with the highest frequency in Soweto (78.9%), followed by Germiston (76.2%), Alexandra (75.0%), Tembisa/Modise (57.1%) and the lowest frequency was detected in Garankuwa (33.3%). The frequency of detection of the antibiotic resistance gene in *C. coli* broth cultures was not statistically significant ($P > 0.05$) (Table 4.8)

Table 4.8: Frequency of six virulence genes, three toxin genes and one resistance gene in *C. jejuni*-positive broth cultures by location of the market

Area[a]	No of positive *C. jejuni* broth cultures	Virulence. n (%)						p-value	Toxin. n (%)			p-value	Total	Antibiotic resistance. n (%)
		Dnaj	*racR*	*flaA*	*flaB*	*ciaB*	*pldA*		*cdtA*	*cdtB*	*cdtC*			*tetO*
Garankuwa	9	2 (22.2)	4 (44.4)	6 (66.7)	5 (55.6)	4 (44.4)	4 (44.4)	0.0006	4 (44.4)	5 (55.6)	4 (44.4)	0.0059	39 (48.1)	4 (44.4)
Tembisa/Modise	6	4 (66.7)	5 (83.3)	2 (33.3)	3 (50.0)	6 (100.0)	6 (100.0)	0.0013	6 (100.0)	6 (100.0)	5 (83.3)	0.0034	43 (79.6)	5 (83.3)
Alexandra	13	9 (69.2)	10 (76.9)	11 (84.6)	11 (84.6)	11 (84.6)	11 (84.6)	0.0001	9 (69.2)	11 (84.6)	10 (76.9)	0.0033	93 (79.5)	4 (30.7)
p-value		0.1383	0.0762	0.1355	0.1189	0.0782	0.0782		0.0488	0.0585	0.0762		0.0784	0.0059
Total	28	15 (53.6)	19 (67.9)	19 (67.9)	19 (67.9)	21 (75.0)	21 (75.0)		19 (67.9)	22 (78.6)	19 (67.9)		174 (69.0)	14 (50.0)

[a]Areas where samples from outlets were positive for *C. jejuni*

Table 4.9: Frequency of six virulence genes, three toxin genes and one antibiotic resistance gene in *C. coli*-positive broth cultures by location of the market

Area[a]	No. of positive *C. coli* broth cultures	Virulence. n (%)						p-value	Toxin. n (%)			p-value	Total	Antibiotic resistance. n (%)
		Dnaj	*racR*	*flaA*	*flaB*	*ciaB*	*pldA*		*cdtA*	*cdtB*	*cdtC*			*tetO*
Garankuwa	9	2 (22.2)	3 (33.3)	7 (77.8)	8 (88.9)	2 (22.2)	2 (22.2)	0.0163	1 (11.1)	3 (33.3)	3 (33.3)	0.0728	31 (38.3)	3 (33.3)
Tembisa/Modise	7	3 (42.9)	2 (28.6)	5 (71.4)	7 (100.0)	3 (42.9)	3 (42.9)	0.0037	4 (57.1)	3 (42.9)	3 (42.9)	0.0099	33 (52.3)	4 (57.1)
Alexandra	4	2 (50.0)	4 (50.0)	3 (75.0)	3 (75.0)	2 (50.0)	0 (0.0)	0.0086	2 (50.0)	2 (50.0)	2 (50.0)	a*	18 (50.0)	3 (75.0)
Germiston	21	10 (47.6)	3 (14.3)	15 (71.4)	20 (95.2)	12 (57.1)	12 (57.1)	0.0034	7 (33.3)	12 (57.1)	10 (47.6)	0.0219	101 (53.4)	16 (76.2)
Soweto	19	5 (26.3)	0 (0.0)	5 (26.3)	7 (36.8)	1 (5.3)	1 (5.3)	0.0421	1 (5.3)	2 (10.5)	4 (21.1)	0.1181	26 (15.2)	15 (78.9)
p-value		0.0429	0.0240	0.0289	0.0354	0.1194	0.1707		0.0581	0.0829	0.0375		0.0497	0.0518
Total	60	22 (36.7)	10 (16.7)	35 (58.3)	45 (75.0)	20 (33.3)	18 (30.0)		15 (25.0)	22 (36.7)	22 (36.7)		209 (38.7)	41 (68.3)

[a]Areas where samples from outlets were positive for *C. coli*

b* = No statistics could be computed because the parameter is constant

Chapter 5: Discussion

The primary health problem posed by chickens from the informal poultry outlets which are spread across the townships in Gauteng province is that they are considered by the government as illegal and therefore unregulated. The poor sanitary practices at these outlets therefore expose the slaughtered chickens to potential human pathogens such as *Campylobacter* species, and *Salmonella* species, which may cause chicken-borne illnesses in consumers of under-cooked, contaminated chickens.

The two frequently isolated species of *Campylobacter* from poultry products and faecal samples of patients with gastroenteritis are *C. jejuni* and *C. coli* (Endtz et al., 1991). However, the prevalence of *Campylobacter* species in chickens sold in the informal poultry market in Gauteng Province, South Africa is not known. Therefore, this study aimed to produce data from the informal poultry market in Gauteng province, South Africa. In five of the visited areas in Gauteng province (Atteridgeville/Phomolong, Garankuwa, Germiston, Soweto and Tembisa/Modise) the outlets had no permanent structure dedicated to the business. Plastics, wood and light metals were used to assemble a structure for the shade and counters where slaughtered chickens are processed or displayed for sale.

The structure used failed to protect processed chickens from dusty conditions which might carry harmful pathogens. The inadequacy of structures at the informal market outlets in Gauteng province in South Africa which may subject chickens from these outlets to contamination by pathogens has been documented for wet chicken outlets in Asia and South America (Tang et al., 2009) and cottage poultry outlets, commonly called ‘pluck shops’ in Trinidad and Tobago (Rodrigo et al., 2006).

The demographic data and risk factors for carcass contamination by *Campylobacter* species obtained from the study reported here revealed how different practices at the sale outlets led to different microbial characteristics of chickens when tested. The absence of facilities has the potential to increase the risk factor for contamination of processed chickens by *Campylobacter* species and other pathogens.

These outlets did not have running water which is a result of their location and the fact that they are illegal. The negative effect of the lack of running water at food processing

outlets has been reported to lead to contamination and cross-contamination of foods by pathogens, such as *Campylobacter* species and *Salmonella* species amongst others (Mosupye and von Holy, 2000, Bryan et al., 1988). Unlike the non-availability of running water at the informal chicken outlets, at the legal chicken retail outlets, running potable water is available and there are ISO standards, guidelines and the hazard analysis critical control point (HACCP) which guide the entire operation so that the food sold to consumers is of good quality and if they are disregarded the whole operation can be shut down (Mosupye and von HOLY, 1999, Mosupye and von Holy, 2000).

The microbial quality of chickens sold to Gauteng consumers was compromised due to the locations of the outlets, which lacked essential resources for the operations of these outlets. Location of these outlets was not the only contributing factor to the poor microbial quality of chickens sold to Gauteng consumers. Poor sanitation and the use of stagnant water were determined to have a high-risk factor for contamination, with the OR of 2.6 and 2.0 respectively.

In this study, majority of the outlets used the stagnant water to rinse processed carcasses covered in blood, feathers and in some cases intestinal contents which came out in the process of evisceration. Rinsing carcasses in this type of system provides a conducive environment for the survival of *Campylobacter* species at ambient temperatures. The nutrient contents of the water are too high therefore increasing the risk of contamination to the next chicken that will be processed, the person processing the chicken and the consumer of the chicken if not cooked properly. In the retail outlets where there are regulations, they use the spray washing or the dipping system to rinse the carcasses after processing which eliminates the chances of biofilm formation that is used by pathogenic microorganisms as a form of protection.

The type of chickens (culled breeders, spent hens or broilers) sold at the informal poultry outlets were also determined to be an important risk factor for the contamination of chicken carcasses by *Campylobacter* species in the current study with high OR of 9.67 and 7.25 detected for culled breeders and spent chickens respectively. Virulence of *Campylobacter* species in chickens increases with age, at the end of their life cycle there is an increase in the number of serovars in the gastrointestinal tract of the chickens which allows for efficient colonization by

Campylobacter species. Therefore, broilers should be the chicken of choice for slaughter at the informal market to minimise the risk of contamination.

In our study, the frequency of isolation of *Campylobacter* species by standard bacteriological methods varied considerably across the six townships/areas from where the samples originated, for example, outlets in Germiston had a statistically significantly higher frequency of *Campylobacter* species isolation (66.7%) than Tembisa/Modise with the lowest frequency (0.0%) (Table 4.4). These differences could be explained, in part, by differences in the practices at these outlets which could affect the prevalence of *Campylobacter* species on chicken carcasses. For chicken outlets in Tembisa/Modise, only broiler chickens were slaughtered, and bucket water was used for rinsing chicken carcasses while in Germiston both culled breeders (OR = 9.67) and spent hens (OR = 7.25) were rinsed in stagnant water in drums. The differences in these practices may have therefore affected the frequency of detection of *Campylobacter* species in chickens from outlets in the two areas. These findings are supported by Kudirkiene et al. (2013) who reported that carcass processing methods (scalding, defeathering and evisceration) compromised the microbial quality of chickens at the outlets because after carcasses were processed the level of contamination increased. In this study we found defeathering of chickens at the sale outlets to be a high-risk factor for *Campylobacter* species contamination (OR = 1.79). All the surveyed outlets used the defeathering method which included either hands only or the knife shaving method which still requires contact of the carcass with the operator's hands.

All the defeathered chickens were scalded, which required chickens to be added to boiling water for easy removal of feathers. This method has been reported in several studies to be of a lesser risk factor compared to other steps involved in processing chickens (Berrang et al., 2001). However, it has been reported that post-scalding, the risk of contamination of chicken carcasses increased primarily because the chicken cannot be processed immediately in its hot state. The chicken is allowed to cool down which makes conditions more favourable for pathogens to grow. This could be pathogens which are on the hands of the operator or those that spread from the neck of the chicken which was slaughtered with an unwashed knife (Berrang et al., 2001).

Berrang et al., 2001 alluded that the increase in *Campylobacter* species isolation after defeathering was caused by contaminated faeces which leaked from the cloaca.

In our study both the conventional bacteriological technique (Lior, 1984) and the conventional PCR method (Persson and Olsen, 2005) were used for the isolation and detection of *Campylobacter* species respectively, from the samples collected in the informal chicken outlets in Gauteng province, South Africa. The prevalence of *Campylobacter* species on chicken carcasses (24.5%) in the current study was significantly lower (P = 0.0091) than that reported for chicken carcasses sampled from the cottage poultry processors 'pluck shops' in Trinidad, 83.9% (Rodrigo et al., 2015) and 32.3% in South Africa (Van Nierop et al., 2005) but higher than 14.7 – 15.0% reported in Korea and Iceland (Lee et al., 2017, Stern et al., 2003). The differences in the prevalence of *Campylobacter* species in chickens reported in various countries may reflect, in part, differences in the conventional bacteriological techniques used in the various studies, varying risk factors for carcass contamination during processing as these have been documented to affect the frequency of detection of the pathogen (Van Nierop et al., 2005). It could also be due to true differences in the carcass prevalence of *Campylobacter* species. To date there is no standard method which is used collectively in different laboratories around the world for isolating *Campylobacter* species from food or veterinary samples which therefore accounts for the variability in the frequencies.

The frequency of isolation of *Campylobacte*r species in cloacal swabs in our study was 37.7% which was lower than 78% reported by Reddy and Zishiri (2018) in Durban, South Africa. It is pertinent to mention that cloacal or caecal carriage of *Campylobacter* species in chickens slaughtered at processing outlets may reflect the carriage rate in chickens on the farms from which they originated (Reddy and Zishiri, 2018). Again, the frequency of cloacal samples positive for *Campylobacter* species varied significantly across the six areas in our study and these findings may also be affected by the types of chickens (broilers, culled breeders or spent hens) which were determined to be risk factors for contamination of carcasses in the informal markets.

The frequency of isolation of *Campylobacter* species in carcass drip in our study was 40.4% which was lower than the 44.4% to 100.0% reported by Rodrigo et al., (2005a) across six health divisions in Trinidad. A statistically significant difference (P = 0.0443)

was noted for the frequency of *Campylobacter* species isolation in carcass drip across the six areas in Gauteng which ranged from 0.0% to 100.0%. The overall isolation rate of *Campylobacter* species was statistically significantly ($P < 0.05$) higher in carcass drips (40.4%) compared to carcass swabs (37.7%) and cloacal swabs (24.5%) This could be explained, in part, by the increased risk of contamination during processing whereby the operators are in contact with the chickens and heavy-duty plastics used for packaging. Most of these processes require hand contact and there is no protective gear or washing of hands with soap to make sure that there is no external source for contamination to the chickens which are on sale. Carcass drip in our study consisted of blood, fats and other proteins which could have easily accumulated in the corners of the heavy-duty plastics and allowed *Campylobacte*r species and other microorganisms to replicate, when the temperature was conducive thus resulting in a higher isolation rate than in the other samples.

It was of diagnostic significance that of the 155 samples determined to be positive for *Campylobacter* species by the conventional bacteriology method only 106 (68.4%) were confirmed to be *Campylobacter* species by conventional PCR and the other 31.6% were therefore considered to be false-positives. It was also significant that the frequency of confirmation of isolates from conventional bacteriology tested by conventional PCR was 70.3% (carcass swabs), 68.4% (cloacal swabs) and 67.2% (carcass drip) (Table 4.5). Identification of *Campylobacter* species has long evolved since the late 1970s, from using the light microscopy to modern techniques used to study the microorganism at a molecular level (Silva et al., 2011).

A direct comparison of the isolation rate by conventional bacteriological method (34.2%) with conventional PCR revealed a statistically significantly ($P < 0.000$) higher detection rate (93.5%) for the three types of samples tested, when applied on enrichment (BB). Equally important was the finding that the conventional bacteriological method detected a statistically significantly ($P < 0.000$) lower prevalence of *Campylobacter* species on chicken carcasses from all sources to be 24.5% (37/151) while the prevalence of detection by the conventional PCR was exceedingly high at 99.2% (127/128). These findings have both diagnostic and food safety relevance. It has been established that PCR is far more sensitive in detecting the DNA of *Campylobacter* species than the conventional bacteriological methods

which detect live organisms. Reports of comparative studies by others have documented increased detection rate from 64.1% to 68.7% when PCR was applied to the same set of samples cultured for *Campylobacter* species (Vinueza-Burgos et al., 2017). These results show that molecular techniques, such as PCR, have the potential to reflect the true prevalence of *Campylobacter* species because they target the DNA, and this allows for the detection of the viable and culturable, viable but non-culturable (VBNC) and non-viable microorganisms (Vinueza-Burgos et al., 2017). It is known that the isolation techniques (enrichment broths, selective solid agar, microaerophilic conditions and identification procedure) significantly affect the recovery of *Campylobacter* species and reported isolation rates for the pathogen (Van Nierop et al., 2005), thus leading to under-reporting of isolation rates. This has the potential to under-estimate the health risk of campylobacteriosis to consumers of undercooked contaminated chickens. The use of the conventional PCR to detect the presence of *Campylobacter* species in chickens and other foods by assaying specifically for the DNA of live and dead *Campylobacter* species has proven to be more efficient than conventional bacteriology. Therefore, although conventional PCR is known to be far more specific than bacteriological methods as demonstrated by its application on the isolates of *Campylobacter* species identified in the current study where conventional PCR confirmed only 68.4% as *Campylobacter* species thus an under-reporting of the prevalence of *Campylobacter* species. In our study, the conventional PCR was also demonstrated to be more sensitive than the conventional bacteriological methods. However, the fact that PCR detects both live and dead *Campylobacter* species (Van Nierop et al., 2005) may limit its accuracy to estimate the food safety risk posed by consumption of under-cooked, PCR-positive chickens.

Other limitations of conventional PCR as a diagnostic tool, include DNA contamination or cross contamination of samples and the presence of inhibitory substances in food if the sample itself is tested or if the enrichment broth (i.e. BB) is screened. This could result in a false-negative/positive reading which have been documented in several studies in the use of conventional PCR as a diagnostic tool (Bartkowiak-Higgo et al., 2006, Van Nierop et al., 2005). In our study, to avoid false-positive results, especially amplicon contamination, different stations were dedicated for preparation of a master mix, addition of the DNA and amplification. Negative and positive controls were included to make sure results were read accordingly to avoid false positives or

negatives. The difference in the isolation and detection of *Campylobacter* species between the bacteriological and PCR methods found in the current study suggest that a combination of both approaches may achieve increased sensitivity and specificity for diagnostic purposes, and equally important, a more accurate assessment of the food safety risk posed by chickens contaminated by *Campylobacter* species.

In the current study, of all the *Campylobacter* species recovered using conventional PCR from positive broth cultures only 22.6% (81/359) were speciated into *C. coli* and *C. jejuni.* Majority of the studies conducted on the detection of *Campylobacter* species in poultry are associated with a higher prevalence of *C. jejuni* than *C. coli.* This was reported in different studies worldwide. In South Africa, Jonker and Picard, 2010 reported on a high prevalence of *C. jejuni* (31.9%) than that of *C. coli* (14.2%) in poultry. In Philippines the frequency of isolation of *C. jejuni* (64.2%) was also reported to be higher than that of *C. coli* (12.1%) (Lim et al., 2017). In the West Indies a number of studies on the informal poultry market reported *C. jejuni* to be more dominant than *C. coli* (Rodrigo et al., 2005a, Rodrigo et al., 2005b, Rodrigo et al., 2006, Rodrigo et al., 2007).

These two species are also associated with cases of campylobacteriosis whereby the same trend was observed, with a higher frequency of *C. jejuni* over *C. coli.* Reports from the literature also show that *C. jejuni* is the dominant species of *Campylobacter* in poultry (Skarp et al., 2016), however, these findings were contrary to the findings made in this study. The dominant species detected was *C. coli* (15.0%) (P = 0.001) as compared with *C. jejuni* (7.5%). Wittwer et al., 2005 suggested that the high prevalence of *C. jejuni* in poultry meat is due to the high genetic diversity which allows it to have a more stable population than *C. coli.* Although these are the general findings from previous studies, there are studies which correlated with the findings in this study (Wittwer et al., 2005).

Van Nierop et al., 2005 reported on the prevalence of these two species in fresh chickens sold by street vendors and *C. coli* (2.0%) had a higher frequency of detection compared with *C. jejuni* (1.0%). In another study from Ecuador the frequency of *C. coli* (18.8%) was higher than that of *C. jejuni* (12.4%) (Vinueza-Burgos et al.,2017). Mabote et al., 2011 also reported a higher prevalence *of C. coli* (48.1%) compared to *C. jejuni* (3.9%) in fresh chickens sold in the informal outlets in North West province,

South Africa. The main reason for the change in prevalence of these two species is still not fully known therefore, more research needs to be done on these two species to understand factors which results in dominance of either *C. coli* or *C. jejuni*.

Although the frequency of *C. coli* was higher than that of *C. jejuni*, this might be a good reflection on the state of the informal poultry market in Gauteng province, South Africa. These two species are predominantly linked to a number of campylobacteriosis cases even though *C. jejuni* is the main culprit, therefore, the high frequency of *C. coli* detection in chicken carcasses may be less of a threat to consumers compared to *C. jejuni.* However, there is a need for further investigation of the predominance of *C. coli* in chicken carcasses contrary to published reports (Mabote et al., 2011; Bester and Essack, 2008; Bester and Essack, 2012)

It is also important to note that only 22.6% (81/359) of the positively detected *Campylobacter* species were speciated as either *C. coli* or *C. jejuni,* the other 77.4% (278/359) were neither *C. coli* or *C. jejuni.* This is an indication that *Campylobacter* species other than *C. coli* and *C. jejuni*, are more prevalent in chickens from the informal chicken market in Gauteng province. The need for further investigation of these other species cannot be over-emphasized.

It is a potential clinical significance that of the six virulence genes and three toxin genes assayed for in the current study, the frequency of detection was significantly ($P < 0.01$) higher in the 27 broth cultures positive for *C. jejuni* than in 54 broths positive for *C. coli.* This did not come as a surprise because *C. jejuni* strains have been associated more frequently with clinical diseases in humans and animals (González-Hein et al., 2013, Datta et al., 2003, Reddy and Zishiri, 2017). The frequency of pathogenic genes detected in this study was 53.8% from 81 speciated broth cultures, with 69.0% detected from 27 *C. jejuni* positive broth cultures and 38.7% detected from 54 *C. coli* positive broth cultures. Different properties contribute to the pathogenesis of *Campylobacter* species and more studies have been conducted on *C. jejuni* which is the predominantly isolated species in gastroenteritis cases (Bessède et al., 2011). In a comparative study on the frequency of virulence genes between humans and commercial chickens in Durban, South Africa, there was a higher frequency of virulence and toxin genes detected in *C. jejuni* isolates (56.3%) compared with the *C. coli* isolates (48.3%) (Reddy and Zishiri, 2018) which agree with the current study. The

high frequency of virulence and toxin genes detected in this study show that *C. coli* and *C. jejuni* have a potential to cause campylobacteriosis in humans. The detection of these genes does not necessarily mean the microorganisms possessing the genes are pathogenic since these genes have to be activated for expression (Carrillo et al., 2004).

We also investigated the possession of resistance genes to tetracycline among *Campylobacter* species isolated from the informal chicken outlets using a duplex conventional PCR to screen for the presence of *tetO* gene. In our study we found that there was a higher frequency of tetracycline resistance in positive *C. coli* broth cultures (68.3%) compared to positive *C. jejuni* broth cultures (50.0%). Tetracycline is not the first drug of choice for clinical therapy of campylobacteriosis in humans, but it is used as an alternative drug. Treatment drugs which are frequently used are macrolides and fluoroquinolones (Endtz et al., 1991). Tetracyclines and gentamycin are used for systemic infections of *Campylobacter* infections (Engberg et al., 2001).

The finding of a high frequency of tetracycline resistance in *Campylobacter* species isolated from chickens sampled in the informal poultry market in South Africa, is a cause for concern. In South Africa, tetracycline has been reported as one of the main antibiotics used as growth promoters in food animals (Eagar et al., 2008) and it has also been documented as one of the frequently used antibiotics in the country (Eagar et al., 2012). The fact that *Campylobacter* species are zoonotic agents indicates that tetracycline-resistant strains of *Campylobacter* species may be transferred to consumers of improperly cooked contaminated chickens and to outlet operators or workers who have direct contact with chicken carcasses during processing, therefore posing zoonotic and therapeutic implications.

In South Africa there is lack of *Campylobacter* Surveillance program which could help in educating and monitoring the use of antibiotics in humans and animals. Various studies have reported on the emergence of *Campylobacter* resistance strains which show that there is a need for concern, especially at the farm level where the farmer is more concerned about making profit and doesn't have enough knowledge on the use of antibiotics.

Limitations

The data base obtained from GDARD on the location and contact numbers of the outlet owners was out dated, making it difficult to locate some outlets in a timely fashion;

A few of the outlet owners were apprehensive about participating in the study because they assumed that we were representing the government until they were convinced with our presentation of the University of Pretoria identification card.

The funds provided by GDARD were inadequate for the projects conducted by several students which caused some delay in conducting the study.

Conclusions

The primary aim of this study was to determine the prevalence, characteristics and risk factors of *Campylobacter* species contamination of chicken carcasses sold at informal poultry outlets in Gauteng province, South Africa. The study concluded that the slaughter of culled breeders and spent hens, defeathering methods, use of stagnant rinse water and poor sanitary practices were significant risk factors for the contamination of chickens slaughtered at the outlets sampled with *Campylobacter* species.

Although the prevalence of *Campylobacter* species on chicken carcasses detected by the conventional bacteriological method was low (24.5%), conventional PCR detected a significantly higher prevalence of 99.2%, indicative of a high contamination rate of chicken carcases with *Campylobacter* species at the informal chicken outlets in Gauteng province. The frequency of detection of *C. coli* in chicken samples tested was significantly higher than *C. jejuni.*

The virulence and toxin genes tested for in the current study were detected at significantly higher frequency in *C. jejuni* isolates compared with *C. coli* isolates, indicative that the *C. jejuni* isolates recovered in the current study pose a higher food safety risk than the *C. coli* isolates. The detection of *tetO* resistance gene in *Campylobacter* broth samples is indicative of the common use of tetracycline in the Gauteng province poultry industry which may have therapeutic implications.

Recommendations

It is recommended that the sanitary practices at the informal chicken outlets in Gauteng province be improved to reduce the risk of contamination of chicken carcasses with *Campylobacter* species and potential chicken-borne campylobacteriosis in consumers. The government needs to acknowledge the existence of the informal market and educate the vendors on how to process chickens, in order to avoid cross-contamination. It will also be helpful if consumers are educated on how to properly cook the chicken. The government should provide designated areas for selling chickens which has cages, a counter for processing, water for rinsing and mobile freezers.

The findings that a majority (76%) of the *Campylobacter* species were neither *C. jejuni* nor *C. coli,* is an indication that other species not detected by PCR may have been present. There is therefore a need to further characterise these samples by PCR.

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Appendix 1

Questionnaire for owners of informal chicken Outlet used during visits to outlets

Serial #:_____ Area**:** _____**Date of Administration**: _____

1. **Name:** _____**(optional)**
2. **Address:** _____**(optional)**
3. **GPS coordinates of outlet:** _____
4. **Contact number(s):** _____
5. **Name of supplier:**
   i. _____ _____ _____ _____
   ii.

	Types of birds slaughtered			
	Spent hens	Culled Breeders	Broilers	Others/combinations (list)
Yes				
No				

   iii. **Approximate number of each chicken type slaughtered daily:**

   a. Spent hens: _____ b. Culled breeders: _____ Broilers: _____

6. **Number of processing days per week:** _____
7. **Key steps in the operation at the outlet (List in sequence):**

Key steps in the operations of the outlets (sequence)			
Serial no.		Yes	No
	Kept chicken in cages		
	Pick chickens for slaughter		
	Slaughter with knives		
	Scalding		
	De-feathering		
	Eviscerate chickens		
	Processing		
	Packaging and retailing		
	Refrigeration		

8. Type of de-feathering method used

	Types of de-feathering method			
	Drum type	Tube type	Hand picking	Others (list)
Yes				
No				

9. Type of rinsing method

	Types of rinsing method		
	Running water/pipe	Stagnant water/Sink/Bucket	Briefly comment on the source of water(tap/river): _____ _____ _____ _____
Yes			
No			

10. Number of years or months in operation: _____

11. Opening hours

	Types of operation

	Weekly	Daily	Briefly comment on the source of water:
Yes			_____
No			_____

12. **Average cost of a whole chicken (Rand**): Spent hens:____, Culled breeders:_____, Broilers:_____

13. **Disposal of wastes from the outlet:**
    a. Liquid waste:_____
    b. Solid waste: _____

14. **Availability of refrigeration or freezing facilities at the outlet (tick**):

	Types of refrigeration method			
	Refrigerator	Freezer	Both	
Yes				
No				

15. **Location of carcass for sale:**
    a. Counter: _____Refrigerator: _____ c. Freezer: _____d. Bucket_____
    b. For outlets where carcasses are located on the counter for sale, how long do they remain there?
        i. Less than 30 min _____ ii. 30 min – 60 min _____ ii. Over 60 min: _____

16. **Number and types of samples collected from the outlet?**
    a. *Chickens:* Spent hens: ____ No.:____ Culled breeders: ___ No.: ___ Broilers: ____ No.: ____
    b. *Cloacal swabs*: Spent hens: ____ No.:____Culled breeders: ___No.: ___Broilers: ___No.:____
    c. Post-evisceration carcass swab: _____
    d. *Rinse water*:_____
    e. *Drip water*: _____
    f. *Whole carcass*_____

17. **Any other comments/observations:**
    i. Do they wash the knives after processing each carcass? Yes.____ No.____

ii. Clean the table with clean cloths? Yes____ No.____
iii. Clean the table with dirty cloths? Yes.____ No.____
iv. Wash hands with soap after processing each carcass? Yes.____ No____
v. Wash hand without soap after processing each carcass? Yes.____ No____
vi. Do not wash hand at all after processing each carcass? Yes.____ No____

18. Type and identification of samples collected at outlet:

Codes

a. Chicken-Spent (CS): _____
b. Chicken-Culled (CC): _____
c. Chicken-Broiler (CB) _____
d. Chicken-Spent Drip (CSD): _____
e. Chicken-Culled Drip (CCD): _____
f. Chicken-Broiler Drip (CB) _____
g. Water rinse/drain liquid in bags with chicken carcass (WR): _____
h. Chicken-Spent Cloaca (CSC): _____
i. Chicken-Culled Cloaca (CCC): _____
j. Chicken-Broiler Cloaca (CBC): _____

19. *Declaration and signature of owner or the person having charge of the animals/birds.*

I, (full name) _____, hereby declare ***that I have consented*** to participate in the study and the samples were collected by the authorized person mentioned above and that no relevant information was withheld from the authorized person.

Date: _____

Appendix 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Isolation and characterization of *Campylobacter spp.* from chickens sold in the informal markets in Gauteng**
PROJECT NUMBER	**V047-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**M Phosa**

STUDENT NUMBER (where applicable)	**S_04896204**
DISSERTATION/THESIS SUBMITTED FOR	**MSc (Tropical Diseases)**

ANIMAL SPECIES	**Slaughtered chickens**	
NUMBER OF SAMPLES	**150**	
Approval period to use animals for research/testing purposes		**May 2017-May 2018**
SUPERVISOR	**Dr. D Morar-Leather**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 23 November 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix 3

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Ms Matshie Phosa
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: (012) 529 8278
Fax: (012) 529 8312
E-mail: darshana.morar@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Ms Phosa,

Your application sent with the email on 27 July 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years

for auditing purposes. A dispensation application may be considered by the Director Animal Health in the event that any of the above is to be stored or distributed;

4. Ethical approval for the study must be obtained from the relevant authority before the study may start.
5. Only chicken carcases from informal markets in Garankuwa, Atteridgeville, Tembisa, Germiston, Alexandra and Soweto, Gauteng Province, may be sampled in this study. No other samples may be obtained from another area without written permission from the Director: Animal Health;
6. Should any imported samples be required for the study, importation of the strains will be subject to obtaining a veterinary import permit prior to the importation.
7. No animal to be used in the study may be purchased or originate from an area under any State Veterinary restriction;
8. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the State Veterinarian of the area. Results of these may not be distributed
9. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
10. This Section 20 approval is valid until 31 December 2018. An application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Isolation and characterisation of *Campylobacter* spp. from chickens sold in the informal poultry markets in Gauteng Province, South Africa.
Researcher: Ms Matshie Phosa
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Our ref Number: 12/11/1/1/8
Your ref:
Expiry date: 31 December 2018

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -10- 31

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Ms Matshie Phosa
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: (012) 529 8278
Fax: (012) 529 8312
E-mail: darshana.morar@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ISOLATION AND CHARACTERISATION OF *CAMPYLOBACTER* SPP. FROM CHICKENS SOLD IN THE INFORMAL POULTRY MARKETS IN GAUTENG PROVINCE, SOUTH AFRICA"

A dispensation is hereby granted on Point 3 of the Section 20 approval that was issued for the above mentioned study (attached):

i) All *Campylobacter* spp. isolates must be stored in the -80°C freezer at the Department of Veterinary Tropical Diseases laboratory, Faculty of Veterinary Science, University of Pretoria.

ii) Stored samples may not be outsourced without prior written approval from DAFF.

iii) Should samples be used for further research, written approval from the Director of Animal Health must be obtained prior to start of project

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017 -10- 31

Appendix 4

Sanitation Score Sheet used for the Study

	Score					
Category	**1 (worst)**	**2**	**3**	**4**	**5 (best)**	**NA***
1. Handlers of chickens at outlet						
1.1 Wore clean clothes with sleeves						
Clean clothes with sleeves						
Clean clothes without sleeve						
Dirty clothes without						
Dirty clothes with sleeves						
Dirty clothes with dirty sleeves						
1.2 Wore aprons						
Wore very clean aprons						
Wore clean aprons						
Did not wear aprons						
Wore moderately dirty clothes						
Wore very dirty aprons						
1.3 Had hair covered						
Yes						
No						
2 Cleanliness in cages or areas where live birds are kept						
Relatively clean and not crowded						
Relatively clean and crowded						
Relatively dirty –faeces etc- and crowded						
Relatively filthy and crowded						
Very filthy and very crowded						
3. Sanitation in slaughter area						
Kept very clean—Little						

blood/feathers/faeces						
Kept clean---some blood/feathers/faeces						
Moderately kept clean—Blood/feathers/lot of flies						
Poorly kept—blood/feathers/lot of faeces/few flies						
Very poorly kept—blood/feathers/faeces/many flies						
4. ***Sanitation in de-feathering or 'plucking' Area***						
Kept very clean—Little blood/feathers/faeces						
Kept clean---some blood/feathers/faeces						
Moderately kept clean—Blood/feathers/lot of faeces						
Poorly kept—blood/feathers/lot of faeces/few flies						
Very poorly kept—blood/feathers/faeces/many flies						
5. Sanitation in evisceration area						
Kept very clean—Little blood/feathers/faeces						
Kept clean---some blood/feathers/faeces						
Moderately kept clean—Blood/feathers/lot of faeces						
Poorly kept—blood/feathers/lot of faeces/few flies						
Very poorly kept—blood/feathers/faeces/ many flies						
6. Sanitation in rinsing of carcases						
Use of 3 rinsing bucket in sequence/Clean water						
Use of 2 rinsing buckets in sequence/Clean water						
Use of 1 rinsing bucket/bloody water/feathers/1carcass						
Use of 1 rinsing bucket/bloody water/feathers/ 2 carcasses						
Use of 1 rinsing bucket/bloody water/feathers/3 or more Carcasses						
7. Sanitation in packaging and sale areas						
Kept very clean—No blood/No feathers/No faeces/No flies						
Kept clean---No blood/ No feathers/No faeces/Few flies						

Moderately kept clean—Some Blood/Few feathers/Some faeces/Some flies						
Poorly kept—blood/feathers/lots of faeces/ few flies						
Very poorly kept—Some blood/Lots of feathers/Lots of faeces/Many flies						

NA: Not applicable

Appendix 5

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V047-17

02 June 2018

Dr D Morar-Leather
Department of Veterinary Tropical Diseases
(darshana.morar@up.ac.za)

Dear Dr Morar-Leather

Project V047-18
Isolation and characterization of *Campylobacter* spp. from chickens sold in the informal markets in Gauteng (M Phoza)

The animal ethics committee notes the inclusion of an additional sample.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa