Use of the *cdt* gene specific PCR in determining virulence properties of *Campylobacter jejuni* isolated from chicken meat samples obtained in some supermarkets in Mafikeng, NWP, South Africa

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Abstract: *Campylobacter* species are implicated as the most common cause of gastroenteritis worldwide and this usually results from the consumption of contaminated water or food. Sporadic cases and /or outbreaks of infections cause by *Campylobacter* species are frequently reported in countries that have more advanced public health facilities. However, in South Africa, chicken processing industries do not perform routine screening for this pathogen. The aim of this study was to isolate *Campylobacter* species from chicken meat samples and to determine their virulent gene determinants using species specific PCR. Chicken samples were bought from some supermarkets, properly labeled and transported on ice to the laboratory for analysis. Samples were analyzed for characteristics of *Campylobacter* using preliminary (Gram staining, oxidase test and catalase test) and confirmatory (API *Campy*) biochemical tests. A total number of seventy (70) preliminary isolates were subjected to the above mentioned tests. The morphology of the species was observed as curved spiral rods that were gram negative. Sixty six (66) isolates were positive for the oxidase test and fifty four (54) isolates for the catalase test. When the confirmatory (API *Campy*) test was used, 62.5% of the isolates were positive for *Campylobacter jejuni* and the rest were identified as *C. lari* and *C. coli*. The subsequent positive isolates were amplified by specific PCR analysis to authenticate identification. The virulent gene determinants *cdtA*, *cdtB*, and *cdtC* were identified using specific PCR and their presence was established. The results obtained in this study indicate that the chicken meat that was tested was contaminated with *C. jejuni*.

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1. Introduction

Campylobacter species are small, curved, motile, microaerophillic gram-negative spiral rods with individual cells range from 0.2-0.9 µm in width and 0.5-5.0 µm in length (Ang et al., 2011). They exhibit rapid, darting motility in corkscrew fashion using a single flagellum (Ang et al., 2011). Although there are many species within the genus, *C. jejuni* and *C. coli* are the most frequently isolated from humans, animals, water and environmental sources (Allos, 2001).

Campylobacter species have been isolated from a wide variety of domestic animals that include pets, cattle, swine, and sheep (Saito et al., 2006; Krutkiewicz and Klimuszko, 2010). However, poultry are known to be the most common source *Campylobacter* and therefore the consumption of improperly cooked poultry meat is the most common risk factor for human infections (Grant et al., 1993; Steinbruecker et al., 2001; Luber et al., 2006; Andersen et al., 2006; Zorman et al., 2006; Han et al., 2007; Aquino et al., 2010; EFSA, 2010; Behringer et al., 2011). *Campylobacter* species cause diseases in humans through the production of toxins and/or invasions and these virulence determinants contribute to survival and establishment of disease in susceptible hosts (Bhavsar and Kapadnis, 2007). Infections caused by *Campylobacter* species include gastroenteritis that may be self limiting (Allos, 2001). However, in some patients the disease may progress to Guillain-Barre Syndrome, a neurological disease that most often results in death (Caporale et al., 2006).

A number of toxins are produced by the different *Campylobacter* species and *C. jejuni* produces the cytolethal distending toxin (CDT) (Lee et al., 2003). CDT causes direct damage on the DNA of the host cell leading to damage. The affected cells arrest in G1 or G2 phases and eventually die. The CDT gene cluster consists of three protein subunits that include the *cdtA, cdtB,* and *cdtC*. Although very little is known about the functions of the *cdtA* and *cdtC* gene fragments, the *cdtB* has recently identified to have nuclease activities (Lee et al., 2003). Beside these virulence determinants, the presence of flagella in pathogenic *Campylobacter* species has been found to enhance attachment, internalization and translocation of epithelial cells of GIT in infected individual and thus contribute to their pathogenicity (Grant et al., 1993; Müller et al., 2011). However, the

ability to resist oxidative stress, toxin production, and invasive properties significantly enhances the survival of *Campylobacter* species in the host and these increase the chances of developing disease (Bhavsar and Kapadnis, 2007).

Several studies have been conducted to determine relationship of *Campylobacter* species from different sources and therefore assess their similarities based on their antibiotic resistance profiles, *flaA*-RFLP, MLST, PFGE and REP-PCR profiles (Luber et al., 2006; Behringer et al., 2011). Close similarities in the antibiotic resistant profiles and genetic patterns between strains obtained from humans and poultry indicating that chicken meat could be a potential source for transmitting *Campylobacter* species to humans (Luber et al., 2006; Behringer et al., 2011). Despite the problems that *Campylobacter* species pose to consumers even in countries with more advanced public health and health care facilities (Andersen et al., 2006; Zorman et al., 2006; Han et al., 2007; Aquino et al., 2010), poultry processing industries do not screen for these pathogens in both live birds and the finished products in South Africa. It is therefore suspected that these pathogens may be transmitted to humans if chicken meat is improperly cooked and consumed. Moreover, in the North West province of South Africa, only one study has been documented that evaluates the occurrence of this pathogen in chicken meat (Mabote et al., 2011). This study was therefore aimed at providing information of both the level of contamination with *Campylobacter* species in chicken meat samples from supermarkets in the Mafikeng and their virulent gene determinants.

2. Materials and methods

2.1 Sampling site

A total of 10 packets of fresh chicken meat samples were bought at supermarkets and butcheries in around Mafikeng.

2.2 Sample collection

The chicken meat samples were properly labeled and transported on ice to the laboratory for analysis. Upon arrival in the laboratory, the samples were analyzed for the presence of *Campylobacter* species within 24 hours.

2.3 Analysis of the samples

Approximately 2g of each chicken meat sample was washed in 5ml of 2% peptone water (Biolab, S.A). Ten fold serial dilutions were prepared and the aliquots of 100µl from each dilution was spread-plated on Brain Heart Infusion (BHI) agar that was supplemented with *Campylobacter* selective supplement (Vacomycin=10mg/L; Polymyxin B=0.25mg; Trimithoprim= 5.0 mg/L) and 5% ox-blood. The selective supplement was obtained from Merck, Germany and supplied by Merck, South Africa. The plates were incubated under micro-aerophilic conditions at 37ºC in 10% CO_2 for 24 to 72 hours. After incubation presumptive *Campylobacter* isolates that were grey in colour were sub-cultured on to fresh BHI agar plates and the plates were incubated as mentioned above. Pure colonies were stored at room temperature until they were identified using preliminary and confirmatory biochemical tests.

2.4 Identification of presumptive *Campylobacter* species

The presumptive *Campylobacter* species were identified using the following criteria:

2.4.1 Gram staining

Isolates were gram stained using the standard methods to determine the morphology of the cells (Cruikshank et al., 1975). Isolates that were Gram negative and spiral in shape were retained for identification using preliminary tests.

2.5 Preliminary Biochemical Tests

2.5.1 Oxidase test

The oxidase test was performed using the oxidase test reagent obtained from Pro-Lab Diagnostics- United Kingdom as indicated in the manufacturer's protocol. Test results were recorded as positive upon the observation of a colour change and negative if no colour was produced.

2.5.2 Catalase test

This test was used to determine the presence of the catalase enzymes that degrade toxic hydrogen peroxide in cells containing the cytochrome oxidase system.

2.6 Confirmatory Biochemical Test

2.6.1 Analytic Profile Index Campy (API Campy)

The API Campy test was performed according to instructions from the manufacturer (BioMériux, Marcy l'Etoile, France). The indices generated for the different isolates were used to determine their identities with the API web software.

2.6.2. Confirmatory identification of *Campylobacter jejuni* using PCR

2.6.2.1 DNA extraction

Pure isolates from the Brain Heart Infusion agar were inoculated into 10ml of nutrient broth and incubated aerobically at 37°C for 24 hours in a shaking incubator to enhance bacterial growth. DNA

was extracted using the alkaline lysis method with certain modifications (Sambrook et al., 1989).

All isolates were screened for identification of *C. jejuni* using *ceuE* specific primers. The primer sequences of the oligonucleotides used are shown in Table 1. All PCR amplifications were performed using DNA thermal cycler (model-PTC-220 DYAD™ DNA ENGINE). The reactions were prepared in 25µl volumes which were made up of 10µg/µl of the template DNA, 50pmol of each oligonucleotide primer set, 1X PCR master mix and nuclease free water. All PCR reagents were obtained from Fermentas, USA but supplied by the Inqaba Biotec Ltd, Sunnyside South Africa. The cycling conditions included an initial DNA denaturation step at 95°C for 5 minutes; 30 cycles of denaturation step at 95°C for 30 seconds; primer annealing at 62°C for 1 minute and primer elongation 72°C for 30 seconds. The final elongation was carried out at 72°C for 10 minutes and PCR products were stored at 4°C.

Table 1: Oligonucleotide sequences of PCR primers that were used for the identification of *Campylobacter jejuni*

Primers	Sequence (5′→3′)	Product size (bp)
JEJ1	CCTGCTACGGTGAAAGTTTTGC	793
JEJ2	GATCTTTTTGTTTTGTGCTGC	

2.6.2.2 PCR for the detection of virulence genes in *C. jejuni* isolates

The virulence gene determinants in *C. jejuni* isolates were determined by specific PCR analysis using the primers that listed in Table 2. Cycling was performed using a DNA thermal cycler (model-PTC-220 DYAD™ DNA ENGINE) at an initial DNA denaturation step at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds; 60°C for 1 minute and 72°C for 30 seconds. The final elongation was carried out at 72°C for 10 minutes and products were stored at 4°C.

Table 2: Oligonucleotide sequences of PCR primers that were used for the detection of virulence genes in *Campylobacter jejuni* isolates

Target gene	Primers	Sequence (5′→3′)	Product size (bp)
cdtA	DS-18	CCTTGTGATGCAAGCAATC	370
	DS-15	ACACTCCATTTGCTTTCTG	
cdtB	*cdtB*-113	CAGAAAGCAAATGGAGTGTT	620
	cdtB-713	AGCTAAAAGCGGTGGAGTAT	
cdtC	*cdtC*-192	CGATGAGTTAAAACAAAAAGATA	182
	cdtC-351	TTGGCATTATAGAAAATACAGTT	

2.6.3 Electrophoresis

The DNA extracted and PCR products were resolved by electrophoresis on a 1% (w/v) agarose gel at 80 volts for 3hours, using 1X TAE buffer (40Mm Tris, 1Mm EDTA and 20 mM glacial acetic acid, pH 8.0). A 100bp DNA molecular weight marker (Fermentas, USA) was included in each gel. The gels were stained in 0.001 µg/ml of ethidium bromide for 15 minutes and the amplicons were visualized under UV light at 420nm wavelength (Sambrook et al., 1989). A gene Genius Bio Imaging System (Syngene, Synoptics; UK) was used to capture the images using Gene Snap (version 6.00.22) software. Gene Tools (version 3.07.01) software (Syngene, Synoptics; UK) was used to analyze the images in order to determine the relative sizes of the amplicons.

2.6.4 Control strain

C. coli (ATCC 43478) and *C. jejuni* (ATCC 33291) were used as negative and positive control strains, respectively, in all experiments.

3 Results

3.1 Detection of *Campylobacter* species using preliminary biochemical and API Campy tests

A total of 70 presumptive isolates identified based on colonial morphology on BHI agar were subjected to preliminary and confirmatory screening tests characteristic of *Campylobacter* species. Results for the different tests are as shown in Table 3. All the isolates were spiral rods. A large proportion of the isolates (54 out of 70) were catalase positive. Further, 66 out of 70 of these isolates were oxidase positive. *Campylobacter* species are both catalase and oxidase positive and these isolates satisfied the identification criterion for the genus. Based on the API Campy test, all the isolates were identified as *Campylobacter* species despite the results obtained for the catalase and oxidase tests. A large proportion of the isolates (61.4%) were identified as *C. jejuni* while *C. coli* and *C. lari* were obtained at proportions of 25.7% and 12.9%, respectively.

Table 3: Results of the biochemical tests and gram staining for identification of *Campylobacter* species

Gram staining	Catalase test		Oxidase test		API Campy		
Spiral rods	+	-	+	-	*C. coli*	*C. jejuni*	*C. lari*
70	54	16	66	4	18	43	9

3.2 Confirmation of *Campylobacter* species using the Polymerase Chain Reaction (PCR) technique

3.2.1 DNA extraction

A total of 70 *Campylobacter* isolates that had been presumptively identified using the API Campy test kit were screened by PCR for characters of *C. jejuni* using specific primers (Table 3). The makeup of these isolates included 18 *C. coli*, 43 *C. jejuni* and 9 *C. lari*. DNA extracted from these isolates is shown in Figure 1.

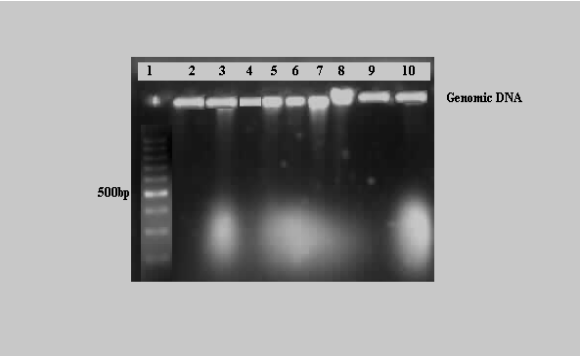

Figure 1: Lane 1= 100bp DNA marker; Lanes 2-10= DNA extracted from *Campylobacter* isolates from test isolates that were screened.

3.2.2 Proportion of *C. jejuni* isolates identified from chicken meat samples obtained from supermarket through PCR amplification of the *JEJ* gene fragments

The identities of *C. jejuni* isolates were investigated based on the presence of the *JEJ* gene fragments using specific PCR analysis. A total number of 70 isolates were screened and a large proportion (71.4%) was identified by PCR as *C. jejuni*. Results obtained from PCR typing indicated that all the isolates that were positively identified as *C. jejuni* using API Campy were also correctly confirmed by specific PCR analysis. However, a small proportion (28.6%) of the *C. jejuni* isolates were not correctly identified by API analysis. From these results it is therefore suggested that molecular methods are more reliable in the detection and classification of microbes in general and these pathogens in particular. Correct identification would facilitate management strategies and thus reduce human infections that result from the consumption of contaminated meat products.

3.2.3 Amplification of the virulent genes from confirmed *Campylobacter jejuni* isolates using specific PCR analysis

The *cdtA*, *cdtB* and *cdtC* virulent gene determinants were amplified from confirmed *C. jejuni* isolates by PCR analysis using specific primer sequences, with *C. coli* (ATCC 43478) and *C. jejuni* (ATCC 33291) serving as negative and positive controls, respectively. A total of 50 *C. jejuni* isolates were subjected to this screening method and the number of isolates that were positive for these genes are shown in Table 4.

Table 4: Number of *Campylobacter jejuni* that were positive for the *cdt* genes

Targeted specie	**No. of virulence genes positive by specific PCR analysis**		
Campylobacter jejuni	*cdtA*	*cdtB*	*cdtC*
NT = 50	18	43	9

4. Discussion

The primary aim of the study was to isolate *Campylobacter* species from chicken meat samples obtained from supermarkets in the Mafikeng area. Although numerous studies have been conducted in countries with more advanced health care systems, very little information exists on the prevalence of *Campylobacter* species in South Africa and Mafikeng in particular (Mabote et al., 2011). In the present study, all the samples were found positive for *Campylobacter* species. The species distribution indicated that *C. jejuni* was more frequently identified when compared to *C. coli* and *C. lari*. These findings are similar to those reported in other studies in which *C. jejuni* and *C. coli* are the species that are mostly isolated from food products such as chicken, eggs, milk and meat (Yu et al., 2001; Saito et al., 2005; Oyarzabal et al., 2007; Rantsiou et al., 2010; Mabote et al., 2011; González and Hänninen, 2011; Kudirkienė et al., 2011). The results obtained in the present study indicated that chicken meat may serve as a potential source for the transmission of *Campylobacter* species to humans. Moreover, *Campylobacter* species are easily transmitted from live birds to humans who work in a broiler house (Shreeve et al., 2000). There is need to screen *Campylobacter* species in the area using genetic fingerprinting methods to facilitate an understanding of the potential transmission routes of this pathogen to human (Steinbruecker et al., 2001). This may reduce the incidence of *Campylobacter* associated infections in humans.

Another objective of the study was to screen positively identified *Campylobacter* species for the presence of the *cdt* virulence gene determinants. *Campylobacter* species cause diseases in humans that result from the ability to express certain virulent genes. The cytolethal distending toxin (CDT) is present in *Campylobacter* species (Johnson and Lior, 1988) and this toxin induces cell distension in different mammalian cell lines resulting in elongation, swelling, and eventually cell death (Whitehouse et al., 1998). Although the *cdt* cluster contains a number of specific gene determinants, in the present study only the *cdt*A, *cdt*B, and *cdt*C were detected. The *cdtA* and *cdtC* are necessary for binding to the host cell while the *cdtB* is the active moiety of the *cdt* ABC complex (Pickett and Whitehouse, 1999; Lara-Tejero and Galán, 2001). The *cdt* genes in *C. jejuni* are known to play a role in

the invasion and modulation of the immune response thereby contributing to the pathogenesis in host cells (Purdy et al., 2000). In chickens the *cdt* genes do not cause inflammation of the intestinal epithelium, while in humans CDT toxin induces the production of neutralizing antibodies which indicates that *C. jejuni* antigens are host-specific (Young et al., 2007). In the present study, all isolates possessed the *cdt* genes and these genes could cause disease in humans if the chicken meat products are consumed undercooked. Moreover, the impact of this pathogen is even more severe when infections are caused by multiple antibiotic resistant strains (Pratt and Korolik, 2005).

Isolation, identification and characterization of *C. jejuni* were carried out using both conventional microbiological and molecular methods, which included polymerase chain reaction (PCR). The results obtained reflected that contamination had occurred in the chicken meat products that were tested and indicated the presence of *Campylobacter* species. However, the contamination identified at retail points do not exclude that which occurred in the farms and the slaughter houses. Contamination during processing can be identified at the retail points or in the finished products (Alter et al., 2006; Kudirkienė et al., 2011). The results therefore indicate that meat products should be cooked properly at a minimum internal temperature of 73.9ºC to prevent these pathogens from producing their virulent gene determinants and thus reduce the occurrence of diseases in humans. This recommendation was proposed by the United States Department of Agriculture – Food safety and inspection services.

Seventy (70) presumptive isolates were screened for characters of *Campylobacter* species using several conventional microbiological methods (Gram staining, oxidase test, catalase test and API Campy test). All the isolates were positively identified. However, *Campylobacter* specific PCR analysis revealed that although a large proportion (71.4%) of those isolates were *C. jejuni*, some of them were false positive results when conventional microbiology methods were employed. It is therefore suggested that to obtain accurate results when investigating the occurrence of pathogens in food and/or water samples, conventional microbiological methods must be used in combination with specific PCR analysis.

5. Conclusions

Campylobacter jejuni was isolated from the chicken meat samples that were bought from various supermarkets and butcheries in the Mafikeng area. Infections that are caused by *Campylobacter* species vary from diarrhoea to the more complicated neuropathological autoimmune diseases such as Guillains Barre Syndrome and Miller-Fisher Syndrome that may lead to the death of the patient (Fry et al., 2000). These infections may be more severe in patients whose immune system is compromised and thus in a country like South Africa the importance of these studies cannot be over emphasized.

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