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Detection of virulent thermophilic *Campylobacter* species in communal chickens

Campylobacter is a foodborne pathogen found in the gut of poultry and other animals, which thereby act as reservoirs for human infection. *Campylobacter* produces various virulence factors which contribute to the establishment of disease. The aim of this study was to detect virulence genes in *Campylobacter* isolated from chicken faeces. A total of 408 poultry cloacal samples were analysed through culture, PCR and DNA sequencing. *Campylobacter* were detected in 24 (5.9 %) of the samples, of which 21 were *C. jejuni* while 3 were *C. coli*. The *cdtB* gene was detected in all *Campylobacter* isolates while *cdtA* and *cdtC* genes were detected in 20 (83.3%) and 18 (75.0%) of the isolates, respectively. The *cadF* gene was detected in 18 (85.7%) *C. jejuni* but not in *C. coli*. Eight genotypes designated G1 to G8 were detected among the isolates. Genotypes G1 and G7 possessed all three *cdt* genes, a potential for producing functionally active toxins. A large proportion (70.8%) of the isolates possessed both genes for toxin production and adhesion. The high prevalence of *cdt* and *cadF* genes in *Campylobacter* species in this study indicates the pathogenic potential of the isolates. There is a need to implement control measures to curb the spread of these virulent strains.

Significance:

- Virulent thermophilic campylobacters cause severe health complications in humans.
- Although chicken meat is the main source of human infections, there is limited data on the occurrence of *Campylobacter* in chicken faeces in southern Africa.
- This study adds to literature on the occurrence of virulent strains of *Campylobacter* species, notably through the deposition of the DNA sequences of our local strains into GenBank®.
- Communal chickens may serve as a source of virulent thermophilic campylobacters to humans, resulting in serious public health implications.

Introduction

Campylobacter species are Gram-negative curved bacteria which normally occur in the gastrointestinal tract of many domestic animals including poultry.¹ These animals serve as potential reservoirs for human infection. If proper farm management techniques are not enforced, *Campylobacter* species can contaminate foods of animal origin and eventually gain access into the food chain.²⁻⁴ Undercooked meat, especially poultry, has been reported to serve as the main vehicle for the transmission of these organisms.^{5,6} Despite the fact that many species have been detected, it is the thermophilic species, most notably *C. jejuni* and *C. coli*, that are responsible for human infection.¹ These species cause diseases in humans ranging from mild self-limiting diarrhoea to severe dysentery and, in some cases, the disease presents with severe complications such as Guillain-Barre Syndrome.⁷⁻⁹

Campylobacter virulence mechanisms include motility, mucus colonisation, toxin production, attachment, internalisation and translocation.¹⁰ These virulence factors and toxins are known to contribute to the survival and establishment of diseases in susceptible hosts.¹⁰ The flagella are used for motility of the bacterium and are known to enhance attachment of the bacterium to epithelial cells.^{7,11} The involvement of the bacterial flagella contributes to their pathogenicity in the host's gut.¹¹ Invasiveness is also an important mechanism that enhances virulence in *Campylobacter* species.¹⁰ These bacteria become internalised into the sub-mucosal cells by means of the flagella and certain proteins.¹⁰ Invasion causes tissue damage and inflammation leading to gastroenteritis.^{10,12}

Campylobacter adhesion to fibronectin (CadF) is an outer membrane protein which aids in the binding of *C. jejuni* and *C. coli* to intestinal epithelial cells,¹³ thus enhancing the colonisation of the gut. Bacterial toxins also play a key role in disease pathogenesis. *Campylobacter* species produce several cytotoxins, notably the cytolethal distending toxin (CDT) which causes direct damage to the DNA of the host cell and eventually leads to cell death.¹⁴ The CDT gene cluster consists of three protein subunits: *cdtA*, *cdtB* and *cdtC*. Although very little is known about the functions of the *cdtA* and *cdtC* gene fragments, *cdtB* has recently been identified to have nuclease activities.¹⁴ However, considering the fact that there are only a few *Campylobacter* genes that have been reported to be directly involved in virulence,¹⁵ the importance of the current study cannot be overemphasised.

Campylobacter is considered to be the most common cause of bacterial gastroenteritis worldwide.¹⁶ The rate of *Campylobacter* infections worldwide has been increasing and it is estimated that incidence of campylobacteriosis exceeds those of salmonellosis and shigellosis.^{17,18} This increase, as well as the expanding spectrum of diseases caused by *Campylobacter* species, necessitates a clearer understanding of the epidemiology and control of campylobacteriosis, especially in developing countries. Campylobacteriosis is common in Africa, especially among children under 5 years, with a prevalence of 7.7–21%.¹⁹⁻²¹

It is worth noting that the agropastoral industry is fast developing in South Africa in particular and in sub-Saharan Africa in general. Unfortunately, animals intended for human consumption are often natural reservoirs for pathogenic bacteria in humans. Infections with thermophilic *Campylobacter* present with severe complications in humans. Despite these health impacts, there are limited data on the prevalence of *Campylobacter* in clinical, environmental and food products in southern Africa.²²⁻²⁵ There is a need to exploit other sources, particularly animal species that are natural hosts of *Campylobacter* species, for the presence of potentially virulent strains.

The present study was designed in order to isolate campylobacters from chicken cloacal samples and to determine the virulence profile of the isolates. Although other genes are responsible for the expression of pathogenicity in *Campylobacter*, the *cdt* gene subtypes (A, B and C) responsible for the expression of the cytolethal distending toxin and the *cadF* gene expressing products of adhesion were selected as targets for the present study.

Materials and methods

Study location

Samples were collected at two poultry farms in Mafikeng, North West Province of South Africa. Laboratory analyses were conducted in the Molecular Microbiology Laboratory of the Department of Microbiology, North-West University, Mafikeng Campus. Ethical clearance for the study was obtained from the Mafikeng Animal Research Ethics Committee (MAREC) of the North-West University, South Africa.

Sample collection

A total of 408 chicken cloacal swabs were collected using the culture swab transport system (COPAN, Italy) and transported on ice to the laboratory for analysis. The samples were analysed for the presence of *Campylobacter* species within 24 h.

Isolation of *Campylobacter* species

Each swab was rinsed in 5 mL of 2% (w/v) sterile peptone water (Biolab, Modderfontein, South Africa) and 100- μ L aliquots spread-plated on *Campylobacter* selective agar supplemented with 5% (w/v) sheep blood and *Campylobacter* selective supplement (Merck, Darmstadt, Germany). The plates were incubated under microaerophilic (10% CO₂) conditions at 42 °C for 24–72 h. Presumptive isolates were sub-cultured and stored in brain heart infusion broth with 20% (v/v) glycerol at -80 °C for further analysis.

Biochemical identification of *Campylobacter* species

All isolates were identified using standard biochemical tests.²⁶ Isolates were Gram stained and observed for Gram-negative spiral rods. The oxidase test was performed using the oxidase test reagent obtained from Pro-Lab Diagnostics (Merseyside, UK) as indicated in the manufacturer's protocol. The catalase test was performed as previously reported.²⁶ The *Campylobacter* test kit (Oxoid, Basingstoke, Hampshire, UK) was used to identify thermophilic campylobacters based on the manufacturer's description.

Molecular characterisation of *Campylobacter* species

Extraction of DNA

Pure isolates were inoculated into 10 mL of nutrient broth (Merck, Darmstadt, Germany) and incubated aerobically at 37 °C for 24 h in a shaking incubator to enhance bacterial growth. Genomic DNA was extracted using the Zymo Research Genomic DNA™ Tissue MiniPrep Kit (Zymo Research Corp, Irvine, CA, USA) based on the manufacturer's instructions and the samples were stored at -20 °C.

Detection of *C. jejuni* and *C. coli*

All isolates were screened for the presence of bacterial 16S rRNA gene fragments and identification of *C. jejuni* and *C. coli* was achieved using the *hipO* and *asp* gene primers, respectively (Table 1). Polymerase chain reaction (PCR) amplifications were performed using the model Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Johannesburg, South Africa). The reactions were prepared in 25- μ L volumes made up of 12.5 μ L of One Taq[®] Quickload 2X Master Mix with standard buffer (Inqaba Biotec Ltd, Pretoria, South Africa), 1 μ L of template DNA, 11 μ L nuclease free sterile water and a final primer concentration of 0.2 μ M. All PCR reagents were obtained from Inqaba Biotec Ltd (Pretoria, South Africa). PCR conditions comprised an initial denaturation at 95 °C for 6 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 40 s and extension at 72 °C for 50 s. A final extension step was performed at 72 °C for 3 min. PCR products were stored at 4 °C until electrophoresis.

Detection of virulence genes

Virulence gene determinants associated with pathogenesis in *Campylobacter* species were determined by specific PCR analysis using the primers listed in Table 1. Cycling was performed using a DNA thermal cycler. The conditions for amplification were the same as those for the identification genes, except for *cdtC* and *cadF* genes, which were annealed at 50 °C.

Table 1: List of primers used for PCR amplification

Primer	Target gene	Sequence 5'-3'	Size (bp)	Reference
Identification genes				
hipO-F	<i>hipO</i>	GACTTCGTGCAGATATGGATGCTT	344	27
hipO-R		GCTATAACTATCCGAAGAAGCCATCA		
CC18F	<i>asp</i>	GGTATGATTCTACAAAGCGAG	500	28
CC519R		ATAAAAGCATATCGTCGCGTG		
27F	16S rRNA	AGAGTTTGATCCTGGCTCAG	1400	29
1492R		TACCTTG TTACGACTT		
Virulence genes				
CDTAF	<i>cdtA</i>	CCTTGTGATGCAAGCAATC	370	30
CDTAR		ACACTCCATTGTCTTTCTG		
CDTBF	<i>cdtB</i>	CAGAAAGCAATGGAGTGTT	620	30
CDTBR		AGCTAAAAGCGGTGGAGTAT		
CDTCF	<i>cdtC</i>	CGATGAGTTAAAAACAAAAGATA	182	30
CDTCR		TTGGCATTATAGAAAATACAGTT		
CADF-F	<i>cadF</i>	TTGAAGGTAATTTAGATATG	400	31
CADF-R		CTAATACCTAAAGTTGAAAC		

Agarose gel electrophoresis

PCR products were resolved by electrophoresis on a 1% (w/v) agarose gel at 80 V for 3 h, using 1X TAE buffer (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid, pH 8.0). A 100-bp DNA molecular weight marker (Fermentas, Glen Burnie, MD, USA) was included in each gel. The gels were stained in 0.001 μ g/mL of ethidium bromide for 15 min and the amplicons visualised using a ChemiDoc Imaging System (Bio-RAD, Hercules, CA, USA).

DNA Sequencing

16S rRNA PCR amplicons were cleaned using a GeneJet PCR Purification kit (Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced at Inqaba Biotec (Pretoria, South Africa). The identities of the isolates were confirmed through a BLAST search of the US National Center for Biotechnology Information (NCBI) database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results and discussion

Bacterial virulence is an important issue that needs scientific investigation. Several virulent determinants are associated with the survival and pathogenesis of *Campylobacter* species. This study was designed to determine the prevalence of *Campylobacter* species in chicken faeces and to identify virulence genes in the isolates. The *cdt* gene subtypes (A, B and C) responsible for the expression of the cytolethal distending toxin and the *cadF* gene expressing products of adhesion were selected for the study.

The occurrence of *Campylobacter* in both clinical and environmental samples from southern Africa has been reported.²²⁻²⁵ Diergaard and colleagues reported the presence of *Campylobacter* spp. in both drinking and environmental water sources in Pretoria.²² In a study conducted in the Limpopo Province, *Campylobacter* species were found to be common causes of gastroenteritis in primary school children.²³ Meanwhile, Uaboi-Egbenni and colleagues detected the presence of *Campylobacter* in

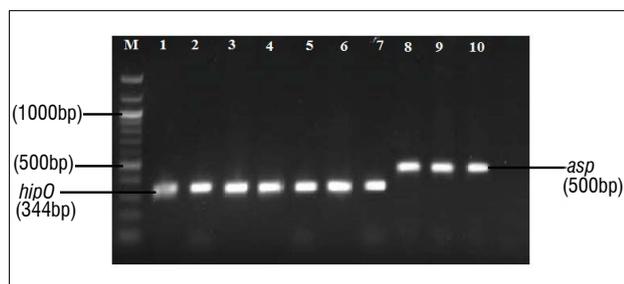
goat faeces.³² In the Mafikeng area, *Campylobacter* has been detected in commercial milk as well as chicken meat.^{24,25} Despite these reports, information on the occurrence of virulent *Campylobacter* in chicken faeces in sub-Saharan Africa is scarce. In the present study, samples were collected from two commercial farms in Mafikeng. These farms sell live chickens to the local population and the chickens are slaughtered at the customers' homes. The handling of live chickens might enhance the spread of enteric pathogens including *Campylobacter* from chickens to humans, especially as standard hygiene practices might not be observed. In such scenarios, the presence of virulent campylobacters in chicken might present a serious public health hazard, more especially as these thermophilic bacteria can survive higher temperatures.

Isolation and molecular identification of *Campylobacter* species

In this study, *Campylobacter* was isolated from 24 of the 408 (5.9%) chicken cloacal samples. However, of the samples inoculated, growth was observed in only 198 (48.5%), and out of this number, 126 (63.6%) were presumptively identified as *Campylobacter* based on growth on selective media and colony morphology. Isolates that presented with the characteristic mucoid cream to pink colonies (Figure 1) were tested using species-specific primers. The isolated DNA was tested for the presence of bacterial 16S rRNA gene fragments and all 126 (100%) presumptive isolates were positive – an indication that the genetic material was all from bacterial sources. Results of the PCR using specific primers showed that only 24 of 126 (19.0%) presumptive isolates were *Campylobacter* species; with 21 (16.7%) and 3 (2.4%) possessing the *hipO* and *asp* genes, respectively, specific for *Campylobacter jejuni* and *Campylobacter coli*, respectively (Figure 2).



Figure 1: Colonies of *Campylobacter* on blood agar.



Lane M, DNA marker (100 base pairs); Lanes 1–7, *hipO* gene of *C. jejuni*; Lanes 8–10, *asp* gene of *C. coli*

Figure 2: Agarose gel of the *hipO* and *asp* genes.

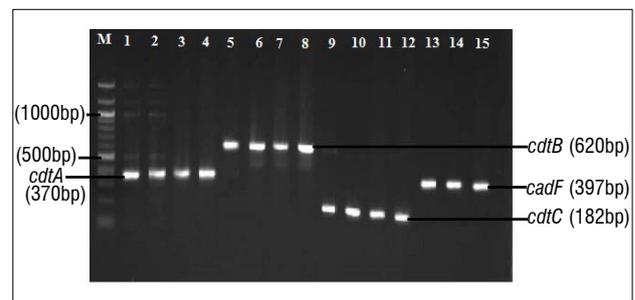
DNA Sequencing

Four isolates that were positive for the *hipO* gene were randomly selected and sent for DNA sequencing to confirm their identities. The identities of the isolates were confirmed through a BLAST search of the NCBI database and all the isolates were confirmed to be *Campylobacter jejuni*. In addition, one of the isolates that grew on the selective media but did not amplify with the campylobacter-specific primers was also sent

for DNA sequencing using the universal 16S rRNA primer. This isolate was found to be *Lactobacillus reuteri*. Similar observations have been recorded in which isolates supposedly identified as *Campylobacter*, were later confirmed by PCR and/or DNA sequencing to belong to other genera. In a related study, Diergaardt et al.²² confirmed by sequencing that only 3 out of 22 presumptive isolates were actually *Campylobacter*. In another study, out of 75 samples examined, only 1 sample (1.33%) was positive by cultural method while 5 samples (6.66%) showed the presence of *Campylobacter* species by PCR.³³ Consequently, these reports confirm the superior efficacy of PCR over culture-based techniques for the rapid screening of both clinical and environmental samples. The sequences of the isolates were deposited in GenBank®, the sequence database of the NCBI, with the following accession numbers: MF872608 (*Campylobacter jejuni*, strain MOLcc14, 99% identity), MF872609 (*Campylobacter jejuni*, strain MOLcc20, 99% identity), MF872610 (*Campylobacter jejuni*, strain MOLcc29, 99% identity), MF872611 (*Campylobacter jejuni*, strain MOLcc30, 99% identity) and MF872612 (*Lactobacillus reuteri*, strain MOLcl43, 99% identity).

PCR detection of virulence gene determinants of *Campylobacter* species

To identify the virulence determinants of *Campylobacter* species isolated from chicken cloacal samples, four genes (*cdtA*, *cdtB*, *cdtC* and *cadF*) were targeted. The *cdt* gene is responsible for the expression of the cytolethal distension toxin which induces cell cycle arrest and promotes DNA damage.^{34,35} Consequently, its presence is associated with the severity of *Campylobacter* disease in humans. Figure 3 shows the amplification of the virulence genes of *C. jejuni*. In this study, the *cdtB* gene was detected in all (24, 100%) *Campylobacter* isolates while *cdtA* and *cdtC* genes were detected in 83.3% and 75% of the isolates, respectively. These results concur with the findings of studies by Gonzalez-Hein et al.³⁶, Carvalho et al.³⁷ and Casabonne et al.³⁸ who detected *cdtB* genes in all *Campylobacter* species isolated from various sources. In contrast, Montwedi and Ateba²⁵ reported relatively lower detection rates of *cdtA* (18/50, 36%) and *cdtC* (9/50, 18%) genes from chicken meat samples. The *cadF* gene encodes a protein that interacts with the host's fibronectin, consequently playing an important role in the adhesion and colonisation of the bacterium in the host's gut. In this study, the *cadF* gene was detected in the majority (18, 85.7%) of *C. jejuni* isolates, in line with previous studies which revealed close to 100% detection of the *cadF* gene.^{36,38,39} However, none of the *C. coli* isolates from the present study showed the presence of the *cadF* gene, possibly because only three *C. coli* isolates were tested. Despite the importance of the *cadF* gene, other genes such as the *flaA* and *ciaB* genes (not investigated in this study) are equally important in the adhesion, colonisation and, consequently, survival of bacteria in the gut.³⁸



Lane M, DNA marker (100 base pairs); Lanes 1–4, *cdtA* gene; Lanes 5–8, *cdtB* gene; Lanes 9–12, *cdtC* gene; Lanes 13–15, *cadF* gene

Figure 3: Agarose gel of the *cdtA*, *cdtB*, *cdtC* and *cadF* gene fragments.

Genotyping of *Campylobacter* species

Genotyping was based on the combination of various identification and virulence genes. A total of eight genotypes designated G1 to G8 were detected among *Campylobacter* isolates (Table 2). G1 was the most prevalent (17, 71.4%) genotype among all *Campylobacter* species. Two genotypes (G1 and G7) possessed all three genes (*cdtA*, *cdtB* and *cdtC*) for toxin expression. These genotypes make up the majority of the



Table 2: Proportion of different genotypes of *Campylobacter* isolates. Genotyping was based on the various combinations of identification (*) and virulence (#) genes.

Genotype	Species	Gene combinations for <i>Campylobacter</i> isolates							Number (%)
		*16rRNA	*hipO	*asp	#cdtA	#cdtB	#cdtC	#cadF	
G1	<i>C. jejuni</i>	+	+	-	+	+	+	+	15 (71.4)
G2	<i>C. jejuni</i>	+	+	-	+	+	+	-	1 (4.8)
G3	<i>C. jejuni</i>	+	+	-	-	+	+	-	2 (9.5)
G4	<i>C. jejuni</i>	+	+	-	-	+	-	-	1 (4.8)
G5	<i>C. jejuni</i>	+	+	-	+	+	-	+	1 (4.8)
G6	<i>C. jejuni</i>	+	+	-	+	+	-	-	1 (4.8)
G7	<i>C. coli</i>	+	-	+	+	+	+	-	2 (66.7)
G8	<i>C. coli</i>	+	-	+	-	+	-	-	1 (33.3)

+, present; -, absent

Campylobacter isolated in this study. The CDT is a tripartite toxin with CDTB being the enzymatically active subunit; however, all three gene products are required for the toxin to be functionally active.³⁴ Consequently, the majority of *Campylobacter* isolated in this study have the potential for producing functionally active toxins. As a result, we envisage a study to investigate the actual expression of toxins by *Campylobacter* isolates from environmental samples. In addition, most of the *Campylobacter* isolates as represented by G1, G3 and G5 possessed both genes for toxin production and adhesion. Interestingly, all the four *C. jejuni* strains that were sequenced belong to G1. The high prevalence of the *cdt* and *cadF* genes in *Campylobacter* species in this study is an indication of the pathogenic potential of the isolates.

Conclusion

Thermophilic campylobacters were successfully isolated from chicken cloacal swabs and the gene sequences of isolates deposited in GenBank. Highly virulent *Campylobacter* species were detected in chicken faeces in the study area, which may present a serious health hazard. Consequently, there is a need to implement control measures to curb the spread of these virulent strains.

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Authors' contributions

M.E.A.B.: Design of methodology, sample collection and laboratory analysis, data validation, writing and revision of the manuscript. C.N.A.: Conception and design of the study, data validation, revision and final approval of the manuscript.

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