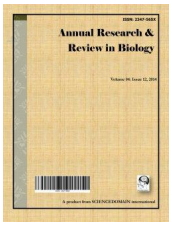

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Molecular Typing of *Campylobacter* Species Isolated from Healthy Indigenous Chickens in Grenada

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VAA, HH and RS designed the study. Authors VAA, VMB and KT managed the sample collection and laboratory analyses. Authors JSH, AS, SKM and SMG performed the molecular analysis. Author VAA managed the literature searches and wrote the first draft of the manuscript. Authors VAA, HH, RS, JSH, SKM and SMG wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To identify the sequence types (STs) of *Campylobacter* from indigenous chickens in Grenada and compare the results to other animals in Grenada and other countries from previous published studies.
Study Design: Thermotolerant *Campylobacter* spp are the leading cause of human gastroenteritis worldwide. Little is known in Grenada about the dynamics of the epidemiology of *Campylobacter* in food animals including indigenous chickens.
Methodology: In a previous study, 158 *Campylobacter* isolates were obtained from cloacal swab

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samples of 315 randomly selected healthy indigenous chickens in Grenada between May and July 2014. After isolation, the 158 *Campylobacter* isolates were stored in 10% sterile skim milk solution at -80°C until ready for DNA extraction. DNA was extracted from only 24 viable *Campylobacter* isolates out of the 158 *Campylobacter* isolates stored. The extracted DNA samples were shipped on dry ice for multilocus sequence typing (MLST) analysis.

Results: A total of 24 viable *Campylobacter* isolates (13 *C. jejuni* and 11 *C. coli*) were identified by polymerase chain reaction (PCR). Different clonal complexes (CCs) and STs were identified from the 24 *Campylobacter* isolates with ST-353 identified as the predominant STs from *C. jejuni* isolates. Most of the previously reported STs in this study belong to ST-828 CC. All the previously reported STs generated from *C. jejuni* have been associated with human gastroenteritis in different geographical regions. Five novel clones which have not been reported in humans or animals worldwide were identified in this study.

Conclusion: This study showed the importance of indigenous chickens as reservoirs for *Campylobacter* species that have been associated with human gastroenteritis worldwide. This study also revealed that indigenous chickens in Grenada harbor novel *Campylobacter* STs that have not been reported in humans and animals worldwide. This is the first report that documents the molecular typing of *Campylobacter* species and the identification of novel *Campylobacter* STs from indigenous chickens of Grenada origin.

Keywords: Campylobacter jejuni; Campylobacter coli; healthy indigenous chickens; Grenada.

1. INTRODUCTION

Campylobacter species particularly *C. jejuni* and *C. coli* have emerged as a major cause of food borne zoonotic illness in humans in many countries during the last two decades [1]. In the poultry industry, three main species (*C. jejuni*, *C. coli*, and *C. lari*) are of the most concern. All three species have been isolated from poultry, but the most pathogenic species in terms of affecting human health is *C. jejuni* which accounts for over 90% of human infection cases, with the majority of the remainder caused by *C. coli* [2,3]. *C. lari* comprises less than 1% of clinical infections in humans [4]. *Campylobacter* infections in humans and animals have been documented in Barbados and Trinidad, both neighboring islands of Grenada [5-8]. In Grenada, there is no published report on the prevalence of *Campylobacter* infections in humans. However, several studies on animals in Grenada have shown that wild and domestic animal species, including poultry [9-11], pigs [12, 13] sheep and goats [14], and mongooses [15] shed *Campylobacter* in their feces.

Indigenous chickens serve as an important food source for the rural poor, benefiting the livelihoods of many individuals in developing countries [16] including Grenada. These chickens mainly feed on food remains, grasses and other wastes, thus exposing them to pathogenic bacteria. Given that indigenous chickens have an even greater chance of contact with pathogenic bacteria, the animals can easily shed the bacterial pathogens into the environment.

Monitoring of *Campylobacter* shedding by animals especially the indigenous chickens is important to understand the epidemiology of *Campylobacter*. Genotyping methods such as multilocus sequence typing (MLST), have been utilized to characterize *C. jejuni* and *C. coli* populations in chickens over the last decade [17].

There are a few reports of *Campylobacter* CCs and STs in wild and domestic animals including small Indian mongooses [15], poultry [10,11], and sheep and goats [14] in Grenada. Some of these CCs and STs have been associated with human gastroenteritis and have been isolated from many animals in different parts of the world suggesting zoonotic transmission. To date, there is no published information on CC or ST of *Campylobacter* isolates from indigenous chickens in Grenada. The goal of this study was to identify the genetic clones of *Campylobacter* from indigenous chickens in Grenada and compare the results to other animals in Grenada and other countries from previous published studies.

2. MATERIALS AND METHODS

2.1 Ethical Approval

The authors hereby declare that “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) were followed. The St. George’s University Institutional Animal Care and Use Committee (IACUC) reviewed and approved all aspects of this project (Approval number: IACUC- 12005-R).

2.2 Sample Collection

In a previous study, 158 *Campylobacte*r isolates were obtained from cloacal swab samples of 315 randomly selected healthy indigenous chickens in Grenada between May and July 2014 [18]. After isolation, the 158 Campylobacter isolates were stored in 10% sterile skim milk solution at -80^{o}C until ready for DNA extraction. DNA was extracted from only 24 viable *Campylobacter* isolates out of the 158 *Campylobacter* isolates stored. The extracted DNA samples were shipped on dry ice for multilocus sequence typing (MLST) analysis.

2.3 DNA Extraction from the Viable *Campylobacter* Isolates

Twenty four viable *Campylobacter* isolates were inoculated into campylobacter-blood-free selective agar (mCCDA) medium (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated microaerobically using Campy-GasPak (BBL Becton Dickson and Co., Cockeysville, Maryland, USA) at 42°C for 48 hours in order to resuscitate the isolates for DNA extraction. DNA were extracted using Qiagen DNAeasy Kit following the manufacturer's instructions (Qiagen Sciences, MD, USA). The extracted DNA from the isolates were shipped to the University of Minnesota Genomic Center (UMGC) (Saint Paul, MN, USA) for MLST analysis. For the identification of *Campylobacter* species, multiplex polymerase chain reaction (m-PCR) was used as described by Denis et al. [19].

2.4 MLST of *Campylobacter* Isolates

To identify the genotypic relationship of the *Campylobacter* isolates from the indigenous chickens and to evaluate similarity to strains associated with human infections, MLST was performed for the 24 *Campylobacter* isolates as previously described [20-24]. Briefly, seven housekeeping genes for *C. jejuni* and *C. coli* (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) were amplified by PCR using the guidelines and PCR primer sequences obtained from the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/mlst-info/Cjejuni/primers.html). PCR was performed using commercial, ready-to-use master mixes [HotStar Taq MasterMix kit (Qiagen, Valencia, CA, USA)] in a Mastercycler (Eppendorf AG), The reaction mixture contained 10 μM of each primer, 12.5 μL of PCR master mix, and 2 μL of DNA template and water to make total volume up to 25 μL. The cycling program consisted of an initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 2 min, 59°C for 1 min annealing, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. To confirm the presence of amplicons of the expected size, PCR amplification products were run on 1.2% agarose gel. Purified PCR products were quantified and sequenced using the same primers as used for PCR amplification. The obtained sequences were analyzed using sequence analysis software (Sequencher ver 5.1, Gene Codes Corporation, MI, USA). Sequence data were submitted to the *Campylobacter* PubMLST database for allele assignments. Allelic profiles were determined by performing BLAST analysis using the single-locus query function, while STs were assigned using the allelic profile query function available in the MLST *Campylobacter* database (http://pubmlst.org/*campylobacter*/). STs were then identified to their respective clonal complexes using BURST at http://pubmlst.org/.

3. RESULTS

Based on PCR analysis, out of the 24 viable *Campylobacter* isolates, 54% (13 of 24) were identified as *C. jejuni* and 46% (11 of 24) as *C. coli*.

Tables 1 shows the allelic profiles and clonal complexes from the *C. jejuni* (n = 13) and *C. coli* (n = 11) identified in this study. Of the 13 *C. jejuni* isolates, four previously reported STs were generated with ST-353 and ST-2927 being the most and least prevalent, respectively. Of the four previously reported STs, ST-353, -5 -50 and -2927 accounted for 38.5%, 23.1%, 15.4% and 7.7% of the 13 *C. jejuni* isolates, respectively (Table 1). Of the 11 *C. coli* isolates, five previously reported STs were generated of which ST-1769, -3839, -5491 accounted for 18.2% (2 of 11) each, and ST-1173, and -8792 accounted for 9.1% (1 of 11) each (Table 1).

Majority of the previously reported STs generated in this study belong to ST-828 CC except for four previous reported STs from *C. jejuni* which are clustered in three different CCs (ST-353 CC, ST-21 CC, and ST-607 CC) (Table 1). Furthermore, the previously reported STs have been associated with human gastroenteritis and identified in other different sources including poultry, ruminants, swine, environments and/or other unspecified sources. They are also distributed in many different geographical regions including USA, Canada, Australia, China, Thailand, Japan, Malawi,

Uruguay, UK, Austria, Belgium, Curacao, Denmark, France, Italy, Germany, Greece, The Netherlands, Lithuania, Latvia, Switzerland, Luxembourg, Spain, Sweden, Estonia, Slovenia, and Portugal (Table 1).

Five novel genetic types (two in *C. jejuni* and three in *C. coli*) were identified in this study (Table 2). The two and three novel clones detected in *C. jejuni* and *C. coli* accounted for 8.3% (2 of 24) and 12.5% (3 of 24) of all the *Campylobacter* isolates, respectively.

4. DISCUSSION

In the present study, the *Campylobacter* genotypes identified by MLST were diverse, with four previously reported STs identified from the *C. jejuni* isolates and five from the *C. coli* isolates. This study also documented that indigenous chickens in Grenada harbor novel *Campylobacter* STs that have not been reported in humans or animals worldwide. In a recent study, 18 previously reported STs and ten novel STs were identified from 99 C*ampylobacter* isolates recovered from pigs in Grenada [24]. Previous studies in commercially reared broiler and layers in Grenada revealed relatively limited *Campylobacter* genotypes with a fewer number of novel STs. In the study by Miller et al. two previously reported STs (ST-3839 and -5491) identified from *C. coli* (Table 1) were unique only to chickens in Grenada [25]. Identification of previously reported potential zoonotic *Campylobacter* STs and novel STs from indigenous chickens of Grenada origin commonly used as source of human food can provide important information on potential sources of infection to humans and possible routes of transmission. Presently, there is no published report on *Campylobacter* of human isolates in Grenada. Thus, there is no substantial evidence that suggest that indigenous chickens may be a source of *Campylobacter* for humans. Continuous monitoring is important in order to determine the risk factor associated with the emergence of novel *Campylobacter* STs in indigenous chickens in Grenada.

4.1 *C. jejuni* ST-353 CC

The *C. jejuni* ST-353 CC identified in this study has been previously reported in Grenada in commercial chickens and sheep [10,11,14]. Of the two STs (ST-353 and ST-5) identified in *C. jejuni* (Table 1), only ST-353 was found in the previous studies in Grenada. Interestingly, both ST-353 and ST-5 have been reported in different countries along with diverse sources (Tables 1) [25] although there is no published information on STs of human isolates in Grenada. Reports from studies carried out more than a decade ago also revealed that the *C. jejuni* ST-353 CC contain isolates associated with human gastroenteritis [26-29]. The isolation of *C. jejuni* ST-353 CC from commercial chickens [10,11], and sheep [14] may suggest that ST-353 is a common CC in Grenada. However, the implication of this ST-353 CC in the epidemiology of *Campylobacter* needs further research.

4.2 *C. jejuni* ST-21 CC

Consistent with our findings, Stone et al. [14] identified the *C. jejuni* ST-21 CC clone in seven out of the 14 *C. jejuni* isolates obtained from sheep and goats. In contrast to their findings in which the ST-21 CC was the most frequent CC observed, this complex appears to be uncommon in indigenous chickens since the complex only occurred in two out of the 13 *C. jejuni* isolates recovered in this study (Table 1). Further, *C. jejuni* ST-21 CC was dominant in dairy calves isolates in Austria [30] and ST-50 clone was among the most prevalent ST isolated. A Luxembourg study [31] indicated that *C. jejuni* ST-21 CC was the most common CC found among human and bovine isolates.

4.3 *C. jejuni* ST-607 CC

Similarly, Stone et al. [11] identified the *C. jejuni* ST-607 CC in commercially raised chickens in Grenada. ST-2927 was the only ST identified from the *C. jejuni* ST-607 CC in this present study as well as the study of Stone et al. [11]. In contrast, the study of Miller et al. [10] did not reveal the presence of *C. jejuni* ST-607 CC in commercial chickens in Grenada. Furthermore *C. jejuni* ST-607 CC was not detected in sheep and goats [14] in Grenada. ST-2927 has been associated with human gastroenteritis also [25].

4.4 *C. coli* ST-828 CC

In the present study, ST-828 CC was the only previously reported CC generated from the *C. coli* isolates (Table 1). Studies have shown that ST-828 CC is one of the most frequent CCs found in humans [25,32]. It has also been identified in ducks [33] and in commercial swine [34]. In comparison with the present study, ST-828 CC was identified in commercial chickens [10,11] but not in sheep and goats [14]

Table 1. Allelic profiles and clonal complexes of *Campylobacter jejuni* and *Campylobacter coli* isolated from indigenous chickens in Grenada**

Allelic profiles	Clonal complex	Sequence type	No. of isolates with ST[GD]	Source	Country[@@]	Record in database
***Campylobacter jejuni* (n = 13)**						
Known	**ST-353**	**5**	**3**	Cow's milk, pig, human stool-sporadic, human unspecified sporadic, chicken, chicken offal/meat, hospital inpatient	UK, Australia, Canada, Netherlands, Thailand, Unknown, Lithuania, Latvia, Switzerland, Luxembourg, Spain, Portugal, Grenada	**436**
	ST-21	**50**	**2**	Chicken, chicken offal/ meat, Environmental waters, human stool-sporadic, human stool-general outbreak human blood culture, human unspecified, potable/ drinking water, lamb, lamb offal, cattle, beef offal, cow milk, turkey, duck, wild bird, dog , sheep, other food, other animal	Austria, Australia, Belgium, Canada, Curacao, Denmark, Italy, Japan, Greece, UK, USA, Spain, China, The Netherlands, Thailand, Switzerland, Sweden, Slovenia, Luxembourg, France, Lithuania, Estonia, Germany, Grenada	**2155**
	ST-353	**353**	**5**	Chicken, chicken offal/meat, environmental waters, human stool-sporadic, human unspecified, human blood culture, hospital inpatient, dog, other animal	USA,UK, Luxembourg, Germany Switzerland, Netherlands, Canada, Japan, Curacao, Estonia, Sweden, Greece, Malawi, Grenada	**201**
	ST-607	**2927**	**1**	Chicken, human stool-sporadic	Thailand, UK, USA	**9**
Novel	**ST-353**	**8784**	**1**	Chicken	**Grenada only**	**2**
	NA[¢¢]	**8777**	**1**	Chicken	**Grenada only**	**6**
***Campylobacter coli* (n = 11)**						
Known	**ST-828**	**1173**	**1**	Chicken, chicken offal/meat, human stool, human unspecified, sheep	USA, Spain, UK, Uruguay, Switzerland, Grenada	**14**
	ST-828	**1769**	**2**	Chicken, goose, human stool- sporadic, human unspecified, other food	UK, Germany, Portugal, Switzerland, USA, Grenada	**16**
	NA[¢¢]	**8792**	**1**	Chicken	UK, Grenada	**2**
	ST-828	**3839[††]**	**2**	Chicken	**Grenada only**	**4**
	NA[¢¢]	**5491[††]**	**2**	Chicken	**Grenada only**	**3**
Novel	**NA[¢¢]**	**8791**	**1**	Chicken	**Grenada only**	**1**
	ST-353	**8788**	**1**	Chicken	**Grenada only**	**2**
	ST-353	**8794**	**1**	Chicken	**Grenada only**	**1**

[**]Information in the PubMLST database retrieved September 6, 2017 [25].
[††] Known STs that are unique only to chickens in Grenada [10, 25].
@@Place(s) where the Campylobacter strains have been recorded
NA[¢¢]Clonal complex or sequence type not assigned as yet
[GD]Isolates recovered from the current study

Table 2. The 5 novel *Campylobacter* clones generated from the 37 *Campylobacter* isolates recovered from indigenous chickens in Grenada**

Species	No. of isolates[GD]	Allele no. for:							Clonal complex	Sequence type
		*asp*A	*gln*A	*glt*A	*gly*A	*pgm*	*tkt*	*unc*A		
C. jejuni	1	2	114	5	2	13	3	6	ST-353	8784
	1	33	176	30	115	113	3	17	NA[¢¢]	8777
C. coli	1	7	2	5	82	113	3	6	ST-353	8794
	1	7	2	300	115	113	3	17	NA[¢¢]	8791
	1	7	17	5	82	113	3	6	ST-353	8788

[**]*Information in the PubMLST database retrieved August 31, 2017 [25].*
NA[¢¢]Clonal complex or sequence type not assigned as yet
[GD]Isolates recovered from the current study

in Grenada. The ST clones identified in commercial chickens differed from those identified in the present study except for ST-3839 and ST-894 identified by Miller et al. [10] and ST-1173 identified by Stone et al. [11]. Rotariu et al. [35] in a study in Scotland indicated that STs of the *C. coli* ST-828 CC were more common in sheep. Overall, the results of this study are in agreement with previous documentation that host association of *Campylobacter* genotypes transcends geographic variation [36].

5. CONCLUSION

In conclusion, the present study revealed the importance of indigenous chickens as reservoirs for *C. coli* and *C. jejuni* STs that have been implicated in human gastroenteritis worldwide. This study also documented that indigenous chickens of Grenada harbor novel *Campylobacter* STs that have not been reported in humans or animals worldwide. To authors' knowledge, this report is the first on identification of novel and various previously reported *Campylobacter* STs from indigenous chickens.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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