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Original Article

Campylobacter Species Isolated from Pigs in Grenada Exhibited Novel Clones: Genotypes and Antimicrobial Resistance Profiles of Sequence Types

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Abstract

Infections caused by *Campylobacter* species pose a severe threat to public health worldwide. However, in Grenada, the occurrence and characteristics of C*ampylobacter* in food animals, including pigs, remain mostly unknown. In this study, we identified the sequence types (STs) of *Campylobacter* from young healthy pigs in Grenada and compared the results with previous studies in Grenada and other countries. Antimicrobial resistance patterns and diversity of the *Campylobacter* clones were evaluated. Ninety-nine *Campylobacter* isolates (97 *Campylobacter coli* and 2 *Campylobacter jejuni*) were analyzed by multilocus sequence typing. Eighteen previously reported STs and 13 novel STs were identified. Of the 18 previously reported STs, eight STs (ST-854, −887, −1068, −1096, −1445, −1446, 1556, and −1579) have been associated with human gastroenteritis in different geographical regions. Among these 18 previously reported STs, ST-1428, −1096, −1450, and −1058 predominated and accounted for 18.2%, 14.1%, 11.1%, and 8.1% of all isolates, respectively. Of the 13 novel STs, ST-7675 predominated and accounted for 20% (4 of 20 isolates), followed by ST-7678, −7682, and −7691, each accounting for 10% (2 of 20 isolates). Antimicrobial resistance testing using *Epsilometer* test revealed a low resistance rate (1–3%) of all *C. coli/jejuni* STs to all antimicrobials except for tetracycline (1–10.1%). Some of the *C. coli* STs (13 STs, 24/99 isolates, 24.2%) were resistant to multiple antimicrobials. This is the first report on antimicrobial resistance and multidrug resistance patterns associated with *Campylobacter* STs recovered from swine in Grenada. This study showed that pigs in Grenada are not major reservoirs for STs of *C. coli* and *C. jejuni* that are associated with human gastroenteritis worldwide.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, sequence type, novel clones, multilocus sequence typing, Grenada

Introduction

Campylobacteriosis is an important public health problem worldwide. It is a zoonosis (Friedman *et al.*, 2000) with most human infections being associated with direct or indirect contact with the animal reservoir. *Campylobacter* infections in humans and animals have been documented in two neighboring islands of Grenada, Barbados (Workman *et al.*, 2005, 2006) and Trinidad (Adesiyun *et al.*, 1992; Adesiyun, 1999). In Grenada, there have been no published reports on the prevalence of *Campylobacter* infections in humans, but studies on animals have shown that both wild and domesticated animals, including pigs (Ganchingco *et al.*, 2012; Matthew-Belmar *et al.*, 2015), sheep and goats (Stone *et al.*, 2014), poultry (Hariharan *et al.*, 2009; Miller *et al.*, 2010; Stone *et al.*, 2013), and mongooses (Miller *et al.*, 2014), shed *Campylobacter* in their feces. There is no published information to date on sequence types (STs) of porcine or human *Campylobacter* isolates in Grenada.

Molecular epidemiology is required to determine the likely sources of human *Campylobacter* infections (Wilson *et al.*, 2008). The multilocus sequence typing (MLST) method uses the relative conservation in sequence of seven housekeeping genes in which variations are likely to be selectively

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neutral (Maiden *et al.*, 1998). MLST analysis has been used to identify *Campylobacter* clonal complexes (CCs) and STs in mongooses (Miller *et al.*, 2014), poultry (Miller *et al.*, 2010; Stone *et al.*, 2013), and sheep and goats (Stone *et al.*, 2014) in Grenada. The objective of this study was to identify the genetic clones of *Campylobacter* from young healthy pigs in Grenada and compare the results with previous studies in other animals in Grenada as well as studies from other countries. The antimicrobial resistance patterns and diversity of the *Campylobacter* clones that infect pigs in Grenada were also evaluated.

Materials and Methods

Sample collection

In a previous study, 172 *Campylobacter* isolates were obtained from 180 pigs of 6 to 12 weeks of age from 14 pig farms within six parishes of Grenada between May and July 2014 (Matthew-Belmar *et al.*, 2015). The 172 *Campylobacter* isolates in the previous study were identified using a biochemical method, which resulted in 92 *Campylobacter jejuni* and 80 *Campylobacter coli* (Matthew-Belmar *et al.*, 2015). Of the 172 *Campylobacter* isolates, 99 isolates were viable for culture and DNA extraction and were identified using polymerase chain reaction (PCR), which is a more reliable method of identifying *Campylobacter* species.

Culture, DNA extraction, and PCR-based identification of viable Campylobacter *isolates*

Ninety-nine viable isolates were inoculated into campylobacter blood-free selective agar (mCCDA) medium (Oxoid Ltd., Basingstoke, England) and incubated microaerobically using Campy GasPak (BBL Becton Dickson and Co., Cockeysville, MD) at 42°C for 48 h to resuscitate the isolates for DNA extraction. DNA was extracted using the Qiagen Dneasy Kit following the manufacturer's instructions (Qiagen Sciences, MD). The extracted DNA from the isolates was shipped to the Veterinary Preventive Medicine Department, Ohio State University, OH, for MLST analysis. For identification of *Campylobacter* species, multiplex polymerase chain reaction was used as described by Denis *et al.* (1999).

MLST of Campylobacter *isolates*

To ascertain the genotypic relationship of *Campylobacter* isolates and to assess similarity to strains associated with human infections, 99 isolates (97 *C. coli* and 2 *C. jejuni*) were analyzed by MLST as described previously (Dingle *et al.*, 2001; Sanad *et al.*, 2011; Kashoma *et al.*, 2014).

Antimicrobial susceptibility testing of Campylobacter *isolates*

The viable 99 *Campylobacter* isolates were tested for susceptibility to seven antimicrobials: ampicillin, tetracycline, erythromycin, ciprofloxacin, gentamicin, chloramphenicol, and metronidazole (AB–Biodisk, Solna, Sweden) by determining the minimum inhibitory concentration (MIC) using the Epsilometer test (*E*-test) strips (AB Biodisk, Solna, Sweden). The *E*-test was performed according to the manufacturer's instructions on Mueller–Hinton agar (Remel) with 5% sheep blood. *C. jejuni* (ATCC 33291) susceptible to all the tested antimicrobials and giving reproducible MICs was used as control (Udayamputhoor *et al.*, 2003). The MIC of a drug was read directly from the scale printed on the *E*-test strip at the point of intersection between the bacterial growth zone and the strip. The breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2016) for *Campylobacter* species were used in the present study, and the MIC values used to classify a strain as resistant were ciprofloxacin, ≥4 μg/mL; erythromycin, ≥32 μg/mL; and tetracycline, ≥16 μg/mL. The National Antimicrobial Resistance Monitoring System–Enteric Bacteria (NARMS) (NARMS, 2012) breakpoints were used when CLSI breakpoints were not available. For chloramphenicol and gentamicin, the breakpoint (≥32 and ≥8 μg/mL, respectively) was used (NARMS, 2012). For ampicillin, the breakpoint (≥32 μg/mL) suggested by Guevremont *et al.* (2006) was used. The breakpoint for resistance to metronidazole was set as ≥16 μg/mL, as recommended by Lorian (1991).

Statistical analysis

The differences in the number of *Campylobacter* isolates showing resistance to multiple antimicrobials and those showing resistance to single antimicrobial as well as the number of isolates showing resistance to the different antimicrobial in this study and previous studies in Grenada were compared using chi-squared (χ^2) (contingency table) analysis created in data analysis plus available in Microsoft Excel 2010 program. A value of $p < 0.05$ was considered statistically significant.

Results

Of the 99 viable *Campylobacter* isolates, 98% (97 of 99) were identified as *C. coli*, while only 2% (2 of 99) were identified as *C. jejuni* as per PCR result obtained during MLST procedure.

Table 1 shows the allelic profiles and CCs from the 99 *Campylobacter* isolates analyzed in this study. A total of 18 previously reported STs were generated from the 99 *Campylobacter* isolates (97 *C. coli* and 2 *C. jejuni*) with eight STs occurring singly and ST-1428 being the most common (Table 1). Of the 18 previously reported STs identified in this study, ST-1428, −1096, −1450, and −1058 predominated and accounted for 16.2%, 14.1%, 11.1%, and 8.1% of all isolates, respectively (Table 1). The common sources and countries where the *Campylobacter* clones obtained in this study have been previously identified are presented in Table 1.

Based on the allelic profiles of the housekeeping genes, 13 novel STs were identified and represent 20 of the 99 isolates (Table 2). Five of the 13 novel STs (STs-7678, −7680, −7686, −7687, and −7688) from this study were assigned to the ST-828 CC. The other eight STs (STs-7673, −7675, −7677, −7681, −7682, −7684, −7685, -and −7691) have not yet been assigned to a CC (Table 2). The only two *C. jejuni* isolates typed in this study belonged to ST-7673. Of the 13 novel STs, ST-7675 predominated and accounted for 20% of the novel isolates, followed by ST-7673, −7678, −7682, and −7691, which accounted for 2 of the 20 novel isolates each. The remaining eight novel STs (ST-7677, −7680, −7681, −7684, −7685, −7686, −7687, and −7688) represented one of the 20 novel isolates each (Table 2).

Table 3 shows the antimicrobial resistance patterns of the *C. coli/jejuni* STs recovered from pigs in Grenada. There was

Table 1. Allelic Profiles and Clonal Complexes from 99 *Campylobacter* Isolates Recovered from Pigs in Grenada

Allelic profiles	*CC*	*Sequence type*	*No. of isolates with ST*	*Source*	*Country*[a]	*Record in database*
Known	ST-828	854	4	Chicken, sheep, pig, human stool, human unspecified, goat, cattle, environmental waters, other animals unspecified	United Kingdom, United States, Spain, Switzerland, The Netherlands, Luxembourg, France, Germany, Italy, Portugal	331
	ST-828	887	1	Chicken, pig, environmental waters, human stool, other animals unspecified	United States, Denmark, United Kingdom, South Korea, Switzerland, Luxembourg, Spain, The Netherlands,	18
	ST-828	1058	8	Pig, chicken, turkey, other animal unspecified	United States, United Kingdom, Switzerland, Germany, Luxembourg, Portugal	21
	ST-828	1068	1	Farm environment, farm slurry, cattle, pig, human unspecified	United States, United Kingdom, Canada	171
	ST-828	1096	14	Pig, chicken, environmental waters, cattle, human unspecified, human stool	United States, United Kingdom, Luxembourg, Germany, Switzerland	76
	ST-828	1124	3	Pig	United States	8
	ST-828	1177	1	Pig	United States	4
	NA[b]	1428	16	Pig	United States	16
	ST-828	1445	2	Pig, human stool	United States, The Netherlands	4
	ST-828	1446	5	Pig, human stool	United States, United Kingdom	8
	NA[b]	1450	11	Pig, environmental waters, ostrich	United States, Luxembourg, United Kingdom	17
	ST-828	1464	1	Pig, other animal unspecified	United States, United Kingdom, China	6
	ST-828	1556	6	Pig, human unspecified, human stool, chicken,	Denmark, Germany, Switzerland, United Kingdom	30
	ST-828	1579	1	Pig, human stool, chicken	United Kingdom, Denmark, Switzerland, Belgium	9
	ST-828	4606	1	Pig	United States	2
	ST-828	4939	1	Pig	Switzerland	8
	ST-828	5746	1	Pig	United States	2
	ST-828	7289	2	Pig	France	3
Novel	[c]	[c]	20	Pig	Grenada only	20

Information in the PubMLST database retrieved January 29, 2016 (*Campylobacter* PubMLST, 2010).
[a]Place(s) where the *Campylobacter* strains have been recorded.
[b]Clonal complex not assigned as yet.
[c]CCs and novel sequence types: presented in Table 2.
CC, clonal complex.

low resistance rate (1–3%) for all the *C. coli* STs to all the tested antimicrobials except for tetracycline (1–10.1%). The *C. coli/jejuni* STs isolated from this study were all susceptible to ciprofloxacin and only two STs, ST-1096 (1%) and ST-1428 (1%), showed resistance to chloramphenicol and erythromycin, respectively (Table 3). The number of isolates showing resistance to tetracycline was significantly higher compared with those showing resistance to the other antimicrobials ($p < 0.001$). Some of the *C. coli* STs (13 STs, 24/99 isolates, 24.2%) were resistant to multiple antimicrobial drugs (MDR to two or more antimicrobial drugs of different classes), while some (13 STs, 42/99 isolates, 42.4%) showed resistance to a single antimicrobial drug. However, four STs (ST-1177, –7680, –7684, and –7687) as well as the two *C. jejuni* (belonging to ST-7673) were susceptible to all the tested antimicrobials (Table 3). Isolates showing MDR were significantly fewer than those showing resistance to one antimicrobial drug ($p = 0.007$). Resistance to tetracycline (61.6%) was the most common resistance observed in this study, followed by metronidazole (16.2%) and ampicillin (13.1%), therefore MDR pattern observed was mainly to tetracycline and other antimicrobial(s). The most common MDR pattern observed was to ampicillin/tetracycline, followed by metronidazole/tetracycline. One isolate, ST-1428, showed MDR to four antimicrobials (ampicillin/erythromycin/metronidazole/tetracycline) and two other isolates belonging to ST-1096 and –7678 showed MDR to three antimicrobials (gentamicin/metronidazole/tetracycline and ampicillin/metronidazole/tetracycline, respectively) (Table 3).

Discussion

The MLST results highlight the *Campylobacter* genotypes found in pigs in Grenada and reveal that certain pig-associated isolates are unique to Grenada pigs. In this study,

Table 2. The 13 Novel Sequence Types Generated from 20 of the 99 *Campylobacter* Isolates Recovered from Pigs in Grenada

Clonal complex	Sequence type	No. of isolates with ST	Allele no.						
			aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
NA[a]	7673[b]	2	53	39	30	81	118	43	36
NA[a]	7675	4	33	38	30	82	118	85	68
NA[a]	7677	1	33	38	44	82	118	35	36
ST-828	7678	2	33	39	30	161	104	43	17
ST-828	7680	1	32	39	30	81	104	35	36
NA[a]	7681	1	32	38	30	82	152	44	17
NA[a]	7682	2	33	39	30	81	104	35	36
NA[a]	7684	1	53	38	30	82	118	43	36
NA[a]	7685	1	53	38	30	81	104	43	36
ST-828	7686	1	33	38	30	85	104	44	17
ST-828	7687	1	53	38	30	82	118	43	17
ST-828	7688	1	33	38	30	82	118	44	17
NA[a]	7691	2	58	38	30	82	104	35	36

Information in the PubMLST database retrieved January 29, 2016 (*Campylobacter* PubMLST, 2010).
[a]Clonal complex not assigned as yet.
[b]Sequence type of the two *C. jejuni* isolates.

Table 3. Antimicrobial Resistance Patterns of the *Campylobacter coli/jejuni* Sequence Types Recovered from Pigs in Grenada

Allelic profile	Clonal complex	Sequence type	*AM*	*CL*	*EM*	*GM*	*MZH*	*TC*	MDR pattern (no. of isolates showing MDR)
			No. (%)						
Known	ST-828	854	1 (1)				1 (1)	3 (3)	AM/TC (1)
	ST-828	887						1 (1)	
	ST-828	1058	2 (2)				2 (2)	6 (6.1)	MZH/TC (2), AM/TC (2)
	ST-828	1068	1 (1)				1 (1)		AM/MZH (1)
	ST-828	1096	1 (1)	1 (1)		1 (1)	3 (3)	10 (10.1)	CL/TC (1), MZH/TC (1), AM/TC (1), GM/MZH/TC (1)
	ST-828	1124						2 (2)	
	ST-828	1177							
	NA[a]	1428	2 (2)		1 (1)		2 (2)	6 (6.1)	AM/TC (1), AM/EM/MZH/TC (1),
	ST-828	1445				1 (1)	1 (1)	2 (2)	MZH/TC (1), GM/TC (1)
	ST-828	1446					1 (1)	5 (5.1)	MZH/TC (1)
	NA[a]	1450						2 (2)	
	ST-828	1464						1 (1)	
	ST-828	1556						3 (3)	
	ST-828	1579						1 (1)	
	ST-828	4606					1 (1)	1 (1)	MZH/TC (1)
	ST-828	4939						1 (1)	
	ST-828	5746	1 (1)						
	ST-828	7289						2 (2)	
Novel	NA[a]	7673[b]							
	NA[a]	7675					2 (2)	2 (2)	MZH/TC (2)
	NA[a]	7677						1 (1)	
	ST-828	7678	2 (2)				1 (1)	2 (2)	AM/TC (1), AM/MZH/TC (1)
	ST-828	7680							
	NA[a]	7681	1 (1)					1 (1)	AM/CIP/TC (1)
	NA[a]	7682	2 (2)					3 (3)	AM/TC (2)
	NA[a]	7684							
	NA[a]	7685						1 (1)	
	ST-828	7686						1 (1)	
	ST-828	7687							
	ST-828	7688					1 (1)	3 (3)	MZH/TC (1)
	NA[a]	7691						1 (1)	
Total			13 (13.1)	1 (1)	1 (1)	2 (2)	16 (16.2)	61 (61.6)	

[a]Clonal complex not assigned as yet.
[b]Sequence type of the two *C. jejuni* isolates.
AM, ampicillin; CL, chloramphenicol; EM, erythromycin, GM, gentamicin; MDR, multidrug resistance (resistance to two or more antimicrobials of different classes); MZH, metronidazole; TC, tetracycline.

most of the isolates belonged to ST-828 CC, which is mainly associated with previously reported isolates from agricultural and environmental sources and human clinical cases (Sheppard *et al.*, 2010b). In Grenada, both Stone *et al.* (2013) and Miller *et al.* (2010) identified *Campylobacter* clones that belonged to the ST-828 CC in poultry; however, none of the STs in this present study was detected in their studies, nor in other animals, including goats and sheep (Stone *et al.*, 2014). Furthermore, researchers in other geographic areas have reported the presence of STs in the ST-828 CC in human, poultry, swine, and cattle (Sanad *et al.*, 2011; Kashoma *et al.*, 2014). There is no published information on STs of human isolates in Grenada. A U.K. study (Roux *et al.*, 2013) indicated that sheep and chicken *C. coli* STs were most frequently found in humans, while those from pigs were rarer. In Denmark, only 10% of the isolates from pigs shared STs with isolates from humans, and these shared STs were found in poultry isolates as well (Litrup *et al.*, 2007). Overall, the results of this study are in agreement with previous documentation that host association of *Campylobacter* genotypes transcends geographic variation (Sheppard *et al.*, 2010a).

Unlike developed countries, Grenada is a small developing island where few individuals own private pig farms and rear pigs in open area for consumption and commercial purposes. The farms are highly disorganized and are in close proximity to humans and livestock. The possibility of intermingling between the pigs and other farm animals exist. Due to the unsystematic distribution of pig farms in Grenada, the relationship between genotype and farms was not determined.

The 18 previously reported STs detected in this study have been identified in swine and other animals as well as humans. ST-854 (331) represents the highest number reported to PubMLST and has been associated with multiple sources, including humans, poultry, ruminants, swine, environment, and other unspecified sources. ST-1068 (171) also represents a high number of reports and has been identified in many sources with ruminants being the highest animal reservoir source (Table 1). ST-1096 (76) has been recovered from numerous sources with swine being the most common animal source, but has not been seen in ruminants. Seven of the STs (ST-1124, –1177, –1428, –4606, –4939, –5746, and –7289) have only been associated with swine as a host species, while ST-1464 has been associated with swine and other unspecified sources (Table 1) (*Campylobacter* PubMLST, 2010).

Eight STs (ST-854, –887, –1068, –1096, –1445, –1446, 1556, and –1579) of the 18 previously reported *C. coli/jejuni* STs recovered in this study have been associated with human gastroenteritis in different geographical regions (Table 1). ST-854, –887, –1096, –1445, and –1579 accounted for 1.3–25% sporadic human gastroenteritis; ST-854, –887, –1068, –1096, –1446, and –1556 accounted for 1.2–12.5% unspecified human infection, while ST-854 accounted for 0.3% hospital inpatient infection (Table 1) (*Campylobacter* PubMLST, 2010).

Based on the results from our antimicrobial susceptibility analysis, resistance of the *Campylobacter* STs to tetracycline (1–10.1%) was predominant and the most common MDR pattern observed was to tetracycline/metronidazole or tetracycline/ampicillin. The high frequency of tetracycline resistance (61.6%) observed in this study differed significantly ($p < 0.001$) from the resistance to tetracycline (19.6%, 10/51) observed in poultry by Stone *et al.* (2013). Another study on sheep and goats in Grenada by Stone *et al.* (2014) revealed resistance of *Campylobacter* isolates to tetracycline (30.8%, 4/13), which is also significantly lower ($p = 0.005$) than the observation in this study. Other studies in different geographical areas have also shown a low resistance rate of *Campylobacter* isolates from different animals and humans to tetracycline in comparison with our observation (Moore *et al.*, 2006; Kashoma *et al.*, 2015). On the other hand, other studies have reported high resistance rates of *Campylobacter* isolates from animals and humans to tetracycline, which is in close agreement with the observation in this study (Hariharan *et al.*, 1990; Rollo *et al.*, 2010; Scott *et al.*, 2012; Stone *et al.*, 2014).

Several studies have shown that tetracycline resistance is common among a variety of bacteria from different sources in Grenada (Sylvester *et al.*, 2014; Amadi *et al.*, 2015a, c, b, d; Farmer *et al.*, 2016). Tetracycline resistance is high in Grenada. It is noteworthy that chlortetracycline is routinely used as a feed additive for pigs in Grenada. Furthermore, oxytetracycline is used for treatment of pigs for bacterial infections (Sabarinath *et al.*, 2011). There is no published information on tetracycline-resistant *Campylobacter* from pigs in other Caribbean islands. Tetracycline resistance was 100% in pig *Campylobacter* in Brazil (Biasi *et al.*, 2011). On the other hand, of seven *C. coli* isolates from pigs, five were resistant to erythromycin, but sensitive to tetracycline (Harrow *et al.*, 2004). The general use of tetracycline has contributed to emergence of resistant bacteria in the environment and clinical sources (Balsalobre *et al.*, 2011).

In conclusion, the present study documents that pigs in Grenada are not major reservoirs for STs of *C. coli* and *C. jejuni* that are associated with human gastroenteritis worldwide. This study revealed that porcine of Grenada origin harbor novel STs that have not been reported in humans or animals worldwide. The resistance rate to drugs other than tetracycline was low. Resistance to erythromycin was very low, but requires continuous monitoring to determine the risk factor for the emergence of erythromycin-resistant *Campylobacter* strains. This is the first report that documents the molecular typing of *Campylobacter* species and resistance and MDR patterns associated with *Campylobacter* STs recovered from swine in Grenada.

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Disclosure Statement

No competing financial interests exist.

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