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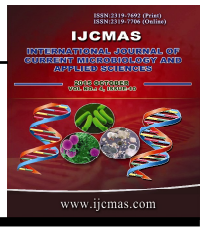

Original Research Article

Antimicrobial Resistance Profiles of *Campylobacter jejuni* and *Campylobacter coli* Recovered from Feces of Young Healthy Domestic Pigs in Grenada

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Keywords

Campylobacter, Antimicrobial resistance, Ampicillin/ tetracycline, Multidrug-resistance, Young healthy, pigs, Grenada

A B S T R A C T

This study determined the prevalence of *C. jejuni* and *C. coli* in feces from 180 randomly selected young, healthy, domestic pigs in Grenada, by culture and their identification and antimicrobial susceptibility profiles by phenotypic characteristics and Epsilometer test, respectively. Fecal samples from 172 of the 180 pigs (95.6%) were culture positive for *Campylobacter* species of which 53.5% were identified as *C. jejuni* and 46.5% as *C. coli*. Out of the 172 *Campylobacter* isolates, 119 viable isolates (65 *C. jejuni* and 54 *C. coli*) were tested for susceptibility against seven antibiotics. Low resistance rate (0 to 3.1%) of all the *C. jejuni* and *C. coli* isolates to four out of the seven antibiotics tested was observed. The highest resistance rates observed was against tetracycline with the resistance rates of 58.5% for *C. jejuni* and 61.1% for *C. coli*; followed by ampicillin, 18.5% for *C. jejuni* and 14.8% for *C. coli*; and metronidazole, 15.4% for *C. jejuni* and 13% for *C. coli*. The ampicillin/tetracycline resistance pattern was the most common pattern for both multidrug-resistant *C. jejuni* (12.3%) and *C. coli* (9.3%). This is the first study to report on multidrug-resistant patterns of *C. jejuni* and *C. coli* isolated from pigs in Grenada.

Introduction

Campylobacter species commonly found in the small intestine of a wide range of wild and domestic animals, including pigs, are the leading bacterial pathogens associated with human and animal bacterial zoonotic gastroenteritis worldwide and thus are of public health concern (Humphrey *et al.*, 2007). They are a major cause of food-borne bacterial diarrhea (CDC, 2014; WHO, 2015) and account for the most commonly reported cases of gastrointestinal infection in humans exceeding salmonellosis and shigellosis (EFSA, 2013). Epidemiological studies have shown an increase in the incidence of human *Campylobacter* infections worldwide. In the United Kingdom, it was estimated that there

were more than a million cases in 2008 and 2009. These cases were associated with approximately 80,000 general practice (GP) consultations (Tam *et al.,* 2012). In 2011, the United States Centers for Disease Control and Prevention (CDC) estimated that there were approximately 845,000 cases of *Campylobacter* infections in the United States each year (Perez-Perez and Kienesberger, 2013), resulting in an annual cost of over a million dollars. In the European Union (EU), the European Food Safety Authority (EFSA, 2013) reported that the cases of campylobacteriosis had increased significantly with 220,209 confirmed cases in 2011. Campylobacteriosis is not a notifiable disease in some parts of the world (EFSA, 2013) especially in the developing countries. Hence cases are greatly underreported and thus the prevalence is underestimated.

Human disease can occur with an infective dose as low as 500 cells of *C. jejuni* (CDC, 2014). Majority of the infections are sporadic and attributed to *C. jejuni* followed by *C. coli* (Piccirillo *et al.,* 2014). However, in some cases, especially in immuno-compromised individuals, infection can spread to the blood stream and advance to life-threatening extra-intestinal infections (i.e. immunoproliferative small intestinal disease) or complications, such as reactive arthritis, Guillain-Barré and Miller-Fisher syndromes (EFSA, 2013). Infected animals are usually asymptomatic carriers (EUFIC, 2006).

Food animals such as pigs, poultry, cattle, sheep and goats are carriers of *Campylobacter* species (WHO, 2015). However, poultry and pigs have been recognized as the major reservoirs of *C. jejuni* (Thakur and Gebreyes, 2005a, 2005b) and *C. coli* (Ghimire *et al.,* 2014; Thakur and Gebreyes, 2005a) respectively. Studies have shown that pathogenic *Campylobacter* species cause disease in pigs (Boosinger and Powe, 1988). In the Caribbean, the prevalence of *Campylobacter* infections in humans and animals have been documented in two neighboring islands of Grenada, Barbados (Workman *et al.,* 2005; Workman *et al.,* 2006) and Trinidad (Adesiyun, 1999; Adesiyun *et al.,* 1992). In Grenada, there have been no published surveys on the prevalence of *Campylobacter* infections in humans. However, previous studies have shown that both wild and domesticated animal species in Grenada, including sheep and goats (Stone *et al.,* 2014), pigs (Ganchingco *et al.,* 2012), poultry (Hariharan *et al.,* 2009; Miller *et al.,* 2010; Stone *et al.,* 2013), and mongooses (Miller *et al.,* 2014) harbor *Campylobacter* species in the gastrointestinal tract. Some of these *Campylobacter* species were resistant to antibiotics that are used in Grenada for the treatment of *Campylobacter* infections in both humans and animals. As the animals in Grenada harbor antibiotic resistant *Campylobacter* species, the animals can shed the resistant organisms into the environment. The emergence and spread of antibiotic resistant zoonotic bacteria constitutes a contemporary worldwide problem that threatens the efficiency of treatment of zoonotic bacterial infections. Thus, continuous monitoring of drug resistance trends in zoonotic bacteria, especially those associated with food animals is necessary prior to making decisions on appropriate steps to reduce drug resistance bacteria in animals and animal products.

The prevalence and antimicrobial resistance patterns of *C. jejuni* and *C. coli* isolates from farmed pigs in Grenada have been previously reported (Ganchingco *et al.,* 2012). Although the prevalence and resistance profile of *C. jejuni* and *C. coli*

recovered from pigs have been documented, continuous monitoring of the resistance profile will reveal any change in the prevalence and the pattern of resistance and provide information on the emerging resistance patterns of *C. jejuni* and *C. coli* to drugs that are currently used for the treatment of *Campylobacter* infections in both human and veterinary clinics in Grenada. The purpose of this study was to determine the current prevalence and antimicrobial susceptibility profiles of *C. jejuni* and *C. coli* recovered from young healthy domestic pigs in Grenada.

Material and Methods

Sample collection: Between May and July 2014, rectal swab samples were obtained from a total of 180 randomly selected young healthy domestic pigs of six to 12 weeks of age, from randomly selected small scale farms from six parishes of Grenada. The sampled pigs did not receive any antibiotics. Thirty rectal swab samples from each parish were collected and stored in a cooler with ice packs and transported to St. George's University, School of Veterinary Medicine, bacteriology laboratory and cultured within three to four hours for the *Campylobacter* species. At the time of sample collection, an identification number, date, age, sex and place of sample collection was recorded.

Culture and phenotypic identification of *Campylobacter* species: Each rectal swab sample was plated on campylobacter-blood-free selective agar (CBF) containing charcoal, cefoperazone and amphotericin B supplements (Oxoid Ltd., Basingstoke, Hampshire, England). The plates were incubated under microaerophilic conditions using Campy-GasPak (BBL Becton Dickson and Co., Cockeysville, Maryland, USA) at 42°C for 48 hours. After incubation, one colony from each CBF plates with characteristics of *Campylobacter* (grey-white, mucoid flat appearance) was subcultured on CBF plate for isolation of pure culture after confirmation of typical morphology of *Campylobacter* by Gram staining. The identification of *Campylobacter* was carried out based on key phenotypic characteristics (Nachamkin, 2003). To speciate the isolates, the cultures were subjected to biochemical tests including catalase, oxidase (BBL, Becton, Dickinson and Co., Sparks, MD, USA), and hippurate tests (Remel, Lennexa, KS, USA), and latex agglutination for *C. jejuni/coli/lari* (JCL, Integrated Diagnostics, Baltimore, MD, USA). All isolates were also tested for their susceptibility to nalidixic acid (30 mg disk) and cephalothin (30 mg disk) (Oxoid) using Mueller–Hinton agar with 5% sheep blood. JCL positive isolates were considered to belong to *C. jejuni/coli/lari* group. Hippurate positive isolates were identified as *C. jejuni*, and nalidixic acid susceptible, hippurate negative isolates as *C. coli*, and nalidixic acid resistant, hippurate negative isolates as *C. lari*. The growths of pure and identified cultures were transferred into 10% sterile skim milk in cryovials, and stored at -85^{o}C until the antibiotic susceptibility testing was carried out. *C. jejuni* (ATCC 33291) was used as control.

Antimicrobial susceptibility testing: The frozen *Campylobacter* isolates were thawed, homogenized, inoculated into CBF (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated microaerophilically using Campy-GasPak (BBL Becton Dickson and Co., Cockeysville, Maryland, USA) at 42°C for 48 hours in order to resuscitate the isolates for the antibiotic susceptibility test. This process yielded only 119 viable *Campylobacter* isolates (*C. jejuni* (65) and *C. coli* (54)) out of the 172 frozen cultures. The viable *Campylobacter* isolates were then tested for susceptibility to seven antibiotics: Ampicillin (AM), Tetracycline (TC), Erythromycin (EM), Ciprofloxacin

(CI), Gentamicin (GM) Choramphenicol (CL), and Metronidazole (MZ) (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 instruction on Mueller–Hinton agar (Remel) with 5% sheep blood as previously described by Hariharan *et al.* (2009). *C. jejuni* (ATCC 33291) susceptible to all the tested antibiotics, and giving reproducible MICs was used as control. 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) for dilution test on aerobic bacteria which was found suitable for *C. jejuni* and *C. coli* by Luber *et al.* (2003) and Guevremont *et al.* (2006) were used in the present study, and the MIC values used to classify a strain as resistant were: ampicillin and chloramphenicol, ≥ 32µg/mL; ciprofloxacin, ≥4µg/mL; erythromycin, ≥ 8µg/mL; gentamicin and tetracycline, ≥ 16µg/mL. The breakpoint for resistance to metronidazole was set as ≥ 16µg/mL as recommended by Lorian (1991).

Results and Discussion

Fecal samples from 180 pigs were examined in this study, of which 172 (95.6%) were culture positive for *Campylobacter* species. Based on our biochemical analysis, all the isolates were positive for oxidase, catalase, and JCL agglutination reactions, and resistant to cephalothin. Of the 172 *Campylobacter* isolates, 53.5% (92 of 172) were identified as *C. jejuni* while 46.5% (80 of 172) were *C. coli*.

Table 1 presents the details of the antimicrobial susceptibility of the 119 viable *Campylobacter* isolates. Overall, our study revealed a low resistance rate (0 to 3.1%) of all the *C. jejuni* (65) and the *C. coli* (54) isolates to four out of the seven antibiotics tested. The highest resistance rate observed was against tetracycline with the resistance rates of 58.5% and 61.1% for *C. jejuni* and *C. coli*, respectively, followed by ampicillin with resistance rates of 18.5% for *C. jejuni* and 14.8% for *C. coli*, and metronidazole with the resistance rates of 15.4% for *C. jejuni* and 13% for *C. coli*. Our results revealed susceptibility of all *C. jejuni* (65) isolates to chloramphenicol and *C. coli* (54) isolates to ciprofloxacin, erythromycin and gentamicin (Table 1).

The multidrug-resistance (MDR – resistance to two or more antibiotics) pattern of the 119 *Campylobacter* isolates is presented in table 2. For *C. jejuni*, six MDR patterns in 19 (29.2%) of the isolates was observed, with ampicillin/tetracycline (12.3%, 8 of 65) being the predominant pattern followed by metronidazole/tetracycline (9.2%, 6 of 65). For *C. coli*, five MDR patterns in 11 (20.4%) of the isolates was observed, ampicillin/tetracycline (9.3%, 5 of 54) being the most common pattern followed by metronidazole/tetracycline (5.6%, 3 of 54).

The present study revealed a prevalence of 95.6% (172 of 180) for *Campylobacter* species in young domestic pigs in Grenada. In a previous study in Grenada, prevalence of *Campylobacter* species was 71.1% (99 of 138) from farmed pigs (Ganchingco *et al.*, 2012). The high prevalence of *Campylobacter* in pigs have also been reported in other countries: Gwimi *et al.* (2015) revealed the prevalence of 92.7% (278 of 300) in Nigeria and Adesiyun *et al.* (1992) reported the prevalence of 79.3% (233 of 294) in Trinidad. In Canada, *Campylobacter* species were also recovered from 99% of grower-finisher pigs as reported by Varela *et al.* (2007). In contrast

to the high prevalence found in our study, a lower prevalence has been reported by others: in the United States, a study carried out by Gebreyes *et al.* (2005) on swine fecal samples revealed a lower rate of 55.8%. In Nepal, Ghimire *et al.* (2014) reported a lower rate of 38.8% in porcine carcass. These differences in reported prevalence of *Campylobacter* in pigs compared to our results could be due to different sampling methods (fecal samples versus carcass swabs), and different bacterial isolation and identification methods. These differences could also reflect true differences in prevalence over time and in different geographic regions.

Based on the phenotypic identification of *Campylobacter* species, the most commonly isolated species in our study was *C. jejuni* (53.5%), with the remainder 46.5% being *C. coli*. Our observations were not in agreement with the results of Ganchingco *et al.* (2012) who identified *C. coli* as the most commonly isolated species (57%) from pigs in Grenada, followed by *C. jejuni* (47%). In Trinidad, Adesiyun *et al.* (1992) also reported a higher prevalence of *C. coli* (64.6%) compared to *C. jejuni* (14.6%). In Nigeria, Gwimi *et al.* (2015) also reported a higher prevalence of *C. coli (*78.7%) compared to *C. jejuni* (14%).

High prevalence rates of *C. coli* in pigs have also been reported by many other researchers worldwide, ranging from 50% to 100% (Aarestrup *et al.,* 1997; Ghimire *et al.,* 2014; Guevremont *et al.,* 2004; Mdegela *et al.,* 2011; Munroe *et al.,* 1983; Payot *et al.,* 2004; Saenz *et al.,* 2000; Steinhauserova *et al.,* 2005; Thakur and Gebreyes, 2005b; Van Looveren *et al.,* 2001; Varela *et al.,* 2007). Although many studies have shown that *C. coli* is the most frequently isolated *Campylobacter* species from pigs, the predominance of *C. jejuni* (53.5%) observed in the present study may be due to the exposure of the tested pigs to *C. jejuni* from sources in Grenada other than pigs which include poultry (Hariharan *et al.,* 2009; Miller *et al.,* 2010; Sharma *et al.,* 2015; Stone *et al.,* 2013), sheep and goats (Stone *et al.,* 2014), mongooses (Miller *et al.,* 2014) and/or the environment (soil and water).

Studies have shown that *C. jejuni* is capable of surviving in the environment despite their fastidious nature, as they can exhibit aerotolerance and starvation survival (Bronowski *et al.,* 2014). We report isolation of only two species (*C. jejuni* and *C. coli)* from pigs in Grenada. Other researchers also report isolation of only *C. jejuni* and *C. coli* in Grenada (Ganchingco *et al.,* 2012) and in Trinidad (Adesiyun *et al.,* 1992). However, Gwimi *et al.* (2015) in Nigeria isolated other *Campylobacter* species from pigs including *C. upsaliensis* (5.4%), and *C. hyointestinalis* (1.8%) using additional selective media.

In the present study, the antimicrobial susceptibility assays revealed a low resistance rate ranging from 0 to 3.1% of all the 65 *C. jejuni* and 54 *C. coli* isolates to four out of the seven antibiotics tested (Table 1). This is in agreement with the findings of Ganchingco *et al.* (2012) who tested *C. jejuni* and *C. coli* isolates from pigs in Grenada to these same antibiotics. Their results revealed low resistance rates as follows: for *C. jejuni*, zero resistance rate to ciprofloxacin, chloramphenicol, erythromycin, and gentamicin; and for *C. coli*, ciprofloxacin (1.8%), chloramphenicol (3.6%), and gentamicin (0%) which are in close agreement with the resistance rates observed in this study (Table 1). However, the resistance rate to erythromycin (8.9%) for *C. coli* reported by these authors (Ganchingco *et al.,* 2012) was higher than the resistance rate to erythromycin (0%) for *C. coli* observed in the current study.

Table.1 Antimicrobial susceptibility profiles of the 119 viable *Campylobacter* isolates recovered in this study as determined by *E*-Test against seven antibiotics

Antibiotic (strip conc.[a] (µg/ml))	*C. jejuni* (n = 65)		*C. coli* (n = 54)	
	Resistance	Susceptible	Resistance	Susceptible
	# (%)**			
Ampicillin (AM-256)	12 (18.5)	53 (81.5)	8 (14.8)	46 (85.2)
Ciprofloxacin (CI-32)	1 (1.5)	64 (98.5)	0	54 (100)
Chloramphenicol (CL-256)	0	65 (100)	1 (1.9)	53 (98.1)
Erythromycin (EM-256)	2 (3.1)	63 (96.9)	0	54 (100)
Gentamicin (GM-256)	1 (1.5)	64 (98.5)	0	54 (100)
Metronidazole (MZH-256)	10 (15.4)	55 (84.6)	7 (13)	47 (87)
Tetracycline (TC-256)	38 (58.5)	27 (41.5)	33 (61.1)	21 (38.9)

**#: Number, % (percentage): values are rounded up and down to one decimal place.
[a]: Strip concentration for all the antibiotics ranged from 0.016 to 256µg/ml except for ciprofloxacin which ranged from 0.002 to 32 µg/ml.

Table.2 Multidrug-resistant patterns of the 119 viable *Campylobacter* isolates recovered in this study as determined by *E*-Test against seven antibiotics

C. jejuni (n – 65)			*C. coli* (n – 54)	
Antibiotics	**# (%)** of isolates**		**Antibiotics**	**# (%)** of isolates**
AM/TC	8 (12.3)		AM/TC	5 (9.3)
EM/MZH	1 (1.5)		TC/CL	1 (1.9)
MZH/TC	6 (9.2)		MZH/TC	3 (5.6)
GM/TC	1 (1.5)		AM/MZH	1 (1.9)
AM/MZH/TC	2 (3.1)		AM/MZH/TC	1 (1.9)
AM/EM/MZH/TC	1 (1.5)			
Total	**19 (29.2)**		**Total**	**11 (20.4)**

**#: Number, % (percentage): values are rounded up and down to one decimal place.
AM = Ampicillin, CI = Ciprofloxacin, CL = Chloramphenicol, EM = Erythromycin, GM = Gentamicin, MZH = Metronidazole, TC = Tetracycline.

High resistance rates to erythromycin for *C. coli* isolated from pigs have been reported in previous studies in different parts of the world with the resistance rates ranging from 39.7% to 92.6% (Aarestrup *et al.*, 1997; Payot *et al.*, 2004; Saenz *et al.*, 2000; Thakur and Gebreyes, 2005b; Van Looveren *et al.*, 2001). The resistance rates to erythromycin (3.1%) and ciprofloxacin (1.5%) for *C. jejuni* observed in the present study are very low. However, the isolation of *Campylobacter* strains exhibiting resistance to these antibiotics are concerning, since the antibiotics are the drug of choice for treating invasive campylobacteriosis cases in humans (Ganchingco *et al.*, 2012; Thakur and Gebreyes, 2005a, b). Furthermore, as the pigs harbor erythromycin and/or ciprofloxacin-resistant *Campylobacter* strains, the animals can easily transfer the resistance gene not only to other *Campylobacter* strains but also to other enteric pathogen of humans and animals and can shed erythromycin and/or ciprofloxacin-resistant *Campylobacter* strains into the environment.

High resistance rates to tetracycline were noted for both *C. jejuni* (58.5%) and *C. coli* (61.1%) in this study. This is in agreement with the high resistance rates (58.1% for *C. jejuni* and 80.4% for *C. coli*) reported by Ganchingco *et al.* (2012). The pattern of tetracycline resistance observed in our study with *C. coli* (61.1%) showing higher resistance rates than *C. jejuni* (58.5%) is in agreement with the pattern observed by Ganchingco *et al.* (2012). Over the last 2 decades, other researchers have observed the same tetracycline resistance pattern in *C. jejuni* and *C. coli* (Hariharan *et al.,* 1990; Rollo *et al.,* 2010; Thakur and Gebreyes, 2005b).

The resistance rate to ampicillin in our study was 18.5% and 14.8% for *C. jejuni* and *C. coli*, respectively. This is in agreement with the resistance rate to ampicillin for *C. jejuni* (14%) reported by Ganchingco *et al.* (2012). However, our results show a higher resistance rate for *C. coli* (14.8%) compared to the 5.4% reported by Ganchingco *et al.* (2012). Because the level of resistance to ampicillin for *C. coli* isolated from pigs in our study have increased slightly compared to the earlier study, continued monitoring of the resistance pattern is necessary in order to identify the risk factors for the emergence of ampicillin-resistant *C. coli* strains in Grenada.

Some of our isolates *C. jejuni* (15.4%) and *C. coli* (13%) showed resistance to metronidazole. The study of Ganchingco *et al.* (2012) revealed a lower resistance rates (2.3% *C. jejuni* and 5.4% *C. coli*) to metronidazole. Although metronidazole is not commonly used against *Campylobacter* infection in humans, metronidazole resistance may serve as a marker for *Campylobacter* species from human diarrheic cases (Andersen *et al.,* 2006).

The present study is the first to report on MDR patterns of *C. jejuni* and *C. coli* isolated from pigs in Grenada. Ampicillin/tetracycline resistance pattern was the most common pattern for both MDR *C. jejuni* (12.3%) and *C. coli* (9.3%).

MDR pattern of *Campylobacter* species of pig origin have been reported in United States and in France by Gebreyes *et al.* (2005) and Payot *et al.* (2004), respectively. Their observations differed from the observations of our study, as erythromycin/nalidixic acid /tetracycline resistance pattern was the most common MDR pattern in their studies.

In conclusion, this study documents the prevalence of antibiotic-resistant *C. jejuni* and *C. coli* in young pigs in Grenada. Among the *C. jejuni* and *C. coli* isolates, the resistance rate to drugs other than tetracycline, ampicillin and metronidazole was very low. Resistance to erythromycin and ciprofloxacin was low but requires continuous monitoring in order to determine the risk factor for the emergence of erythromycin and ciprofloxacin-resistant *Campylobacter* strains since these antibiotics are used in the treatment of severe invasive cases of campylobacteriosis.

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