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Prevalence and Antimicrobial Resistance of *Campylobacter* Species Isolated from Backyard Chickens in Grenada, West Indies

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Authors' contributions

This work was carried out in collaboration between all authors. Author RS designed the study, oversaw the research and wrote the first draft of the manuscript. Author HH supervised the laboratory work and reviewed the manuscript. Authors KT, VMB, VAA and NW performed specimen collection, isolation of cultures, antimicrobial drug testing and data analysis. Authors SMG and SK performed PCR on cultures and review of final draft of the manuscript.

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ABSTRACT

Aim: This study was carried out to assess the prevalence of *Campylobacter* spp. in free range chickens in Grenada, West Indies and to determine the antimicrobial susceptibility of isolates.
Study Design: Cloacal swabs were collected from 315 free range chickens from randomly selected households from all six parishes of Grenada between June and July 2014. Cloacal swabs were cultured for *Campylobacter* in the Pathobiology Diagnostic Laboratory, School of Veterinary medicine, St. Georges University Grenada. Isolates were further tested through PCR for speciation.

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Methodology: Standard culture methods for isolation of *Campylobacter* spp were used. Isolates were characterized by conventional phenotypic tests and confirmed by PCR using species specific primers. The 16s rRNA gene for *Campylobacter* spp.; the *map A* gene for *C. jejuni* and the *ceuE* gene for *C. coli* were selected for PCR. Isolates were tested through E-test for their antimicrobial susceptibility using Ampicillin, Ciprofloxacin, Chloramphenicol, Erythromycin, Gentamicin, Metronidazole and Tetracycline.
Results: A total of 158 isolates (50.2%) were obtained by culture. PCR on 93 isolates identified 33 isolates as *C. jejuni,* 6 as *C. coli* and 54 as mixed infection with *C. jejuni* and *C. coli.* None of the isolates was resistant to chloramphenicol and erythromycin; susceptibility to other antimicrobials varied among isolates. Multidrug resistance was high in *C. coli (33.3%),* followed by mixed infection isolates (22.2%) and *C. jejuni (12.0%).*
Conclusion: Results of the study show that approximately 50% of backyard chickens in Grenada harbor *Campylobacter* spp. These backyard chickens pose a great risk for humans as hazard analysis critical control point (HACCP) is not observed during the slaughter and processing of these chickens.

Keywords: Campylobacter spp.; backyard chickens; antimicrobial testing; Grenada (West Indies).

1. INTRODUCTION

Campylobacter is recognized as the most important zoonotic pathogen in both developed and developing nations of the world [1,2]. *Campylobacter* colonizes intestine tracts of animals and birds and is excreted in feces. Meat of animals gets contaminated by intestinal contents of infected animals during careless slaughter process. Humans get infected by handling animals and birds colonized by *Campylobacter* in their intestines, consumption of *Campylobacter* contaminated meat, or ingestion of contaminated food and water [3].

In developing countries, people rear backyard chickens for food and income [4]. Grenada is a small island in the southeastern Caribbean with 344 km^2 land size [5]. Approximately 30% of households in Grenada rear approximately 26,000 backyard chickens on the island (Dr. Bowen Louison, Chief Veterinary Officer, Ministry of Agriculture, Lands, Forestry, Fisheries and Environment, Grenada: Personal communication). The flock size in each household ranges from 5 to 40.

Information on *Campylobacter* infection in animals and birds in Caribbean islands is scanty. The isolation of *Campylobacter* from food animals, dogs and chickens has been reported from Trinidad [6] and Barbados [7], both neighboring islands of Grenada. Exhaustive research conducted in Grenada revealed the presence of *Campylobacter* in healthy pigs [8], sheep and goats [9], and commercial broilers and layers [10,11]. To authors' knowledge there is no published report on the isolation of *Campylobacter* from backyard chickens in Grenada or other Caribbean islands. In the present study, we investigated the prevalence in Grenada of *Campylobacter* in backyard chickens followed by antimicrobial susceptibility of the isolates.

2. MATERIALS AND METHODS

2.1 Ethical Approval

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. All aspects of the project were examined and approved by the Institutional Animal Care and Use Committee (IACUC) of the St. George's University (Approval number-IACUC-12005-R).

2.2 Birds and Sampling

Three hundred fifteen backyard chickens selected randomly from all six parishes were included in the study after obtaining verbal permission from bird owners. Cloacal swabs were collected from these birds using sterile swabs and Cary Blair transport medium (BBL Beckson and Co. Cockeysville, Maryland, USA). The cloacal swabs were transported within 4 h. on ice to Pathobiology Research laboratory, School of Veterinary Medicine, St George's University.

2.3 Culture and Identification of *Campylobacter*

Method described by Hariharan et al. [10] was followed for bacterial culture. The cloacal swabs were plated on *Campylobacter* blood free

selective agar (CBF) containing charcoal, cefoperazone and amphotericin B supplement (Oxoid Ltd, Basingstoke, Hampshire, England). The plates were incubated at 42℃ for 48 h. in anaerobic jars under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen) using campy gas generating pack (BBL Becton Dickson and Co. Cockeyville, Maryland USA). The grayish non translucent colonies grown on plates were stained with Gram's stain and examined under a microscope at 1000X magnification for typical Gram negative gull-shaped morphology of *Campylobacter.* Positive colonies were sub-cultured on CBF for purification. *Campylobacter* isolates were stored in 10% skim milk at -80℃ for further research [12]. Methods for culture identification and speciation were those described by Nachamkin [13]. Briefly, fresh cultures were tested for catalase and oxidase reactions (BBL Becton, Dickinson and Co. Sparks, MD, USA) and hippurate tests (Remel, Lennexa, KS, USA). Cultures were also tested for their susceptibility to nalidixic acid (30 µg disc) and cephalothin (30 µg disc) on Mueller- Hinton agar with 5% sheep blood. Hippurate positive isolates were identified as *C. jejuni* and hippurate negative and nalidixic acid susceptible isolates as *C. coli. C. jejuni* (ATCC33291) was used as control.

2.4 DNA Extraction and PCR Based Identification of *Campylobacter*

Genomic DNA was extracted from enriched broths of the isolates using DNeasy blood and tissue kit (Qiagen, USA) following the manufacturer's instructions. Two separate PCRs were conducted for screening of 93 isolates of *Campylobacter* following the methods described by Denis et al. [14]. A Multiplex PCR was performed by targeting 16S rRNA gene for *Campylobacter* genus and *mapA* gene for *C. jejuni*. Another individual PCR was performed by targeting *ceuE* gene for *C. coli.* The primers used in PCR for *Campylobacter* species and the size of amplified fragments are presented in Table 1.

PCR amplifications (both multiplex and individual) were carried out using a 25 µl reaction mixture containing 12.5 µl of the master mix, 1 µl of each primer (10 µM), and 2 µl of purified DNA (containing approx. 20 ng of DNA) and the final volume was made up to 25 µl by adding dH_2O. The conditions for both multiplex PCR and the individual PCR were as follows: initial denaturation at 95℃ for 15 min followed by 35 cycles of 1 min denaturation at 95℃, 1 min of annealing at 48℃, and 1 min of extension at 72℃, and a final 10 min extension at 72℃ after the last cycle. PCR products at 10 µl were subjected to electrophoresis with 1.5% agarose gel, stained with ethidium bromide, and photographed under gel documentation system (LabNet International Inc.).

2.5 Antimicrobial Susceptibility Testing

Campylobacter isolates were tested for antimicrobial susceptibility using the Epsilometer test (E-Test) strips (AB Biodisk, Solna, Sweden) following manufacturer's instructions. Fresh bacterial cultures adjusted to MacFarland No. 1 turbidity standard using sterile distilled water were plated on Mueller-Hinton agar with 5% sheep blood. After applying E-test strip, the cultured plates were incubated for 24 h. at 42℃ in microaerophilic condition using a Campy pack (Oxoid). The MIC of the 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, according to manufacturer's instructions. The interpretation of MIC was based on the description of Luber et al. [15]. *C. jejuni* (ATCC33291) susceptible to all tested antimicrobials and given reproducible MICs was used as control. The MIC values to classify a strain as resistant were: ampicillin and chloramphenicol ≥32 µg/ml; ciprofloxacin ≥4 µg/ml; erythromycin ≥8 µg/ml; gentamicin and tetracycline ≥16 µg/ml; and breakpoint for metronidazole was set at ≥16 µg/ml as per Lorian [16].

Table 1. Primers used for *Campylobacter* species

Name		Sequence		Size of amplified fragment	Reference of primers
Campylobacter Genus (CG)	F 5'	ATCTAATGGCTTAACCATTAAAC	3'	857	Denis et al. [14]
	R 5'	GGACGGTAACTAGTTTAGTATT	3'		
Campylobacter jejuni (CJ)	F 5'	CTATTTTATTTTTGAGTGCTTGTG	3'	589	
	R5'	GCTTTATTTGCCATTTGTTTTATTA	3'		
Campylobacter coli (CC)	F 5'	AATTGAAAATTGCTCCAACTATG	3'	462	
	R 5'	TGATTTTATTATTTGTAGCAGCG	3'		

3. RESULTS

A total of 315 backyard chickens were examined for *Campylobacter* spp. Of these, 158 (50.2%) were positive for *Campylobacter*. Of the 158 isolates, only 93 were obtained in a viable form. These isolates were further tested by PCR for speciation. The results are presented in Table 2. Mixed infection with *C. jejuni* and *C. coli* was highest (58.1%) followed by *C. jejuni* (35.5%) and *C. coli* (6.5%).

Antimicrobial resistance determined by E-Test against seven drugs on 93 isolates of *Campylobacter* revealed 0% resistance to chloramphenicol (CL) and erythromycin (EM). Results of antimicrobial resistance are presented in Table 3.

Multiple drug resistance (Table 4) was the maximum in *C. coli* strains (33.3%) followed by mixed infection with *C. jejuni* + *C. coli* (22.2%) and then *C. jejuni* (12.0%).

Table 2. *Campylobacter* isolates identified by PCR from backyard chickens in Grenada

Isolates on culture and PCR	Percentage of positive samples
Campylobacter isolates on culture	158/315 (50.2%)
C. jejuni on PCR	33/93 (35.5%)
C. coli on PCR	6/93 (6.5%)
C. jejuni and C. coli (mixed infection) on PCR	54/93 (58.0%)
Campylobacter genus on PCR	93/93 (100%)

Table 3. Antimicrobial resistance patterns of *Campylobacter* isolates from backyard chickens in Grenada

Antimicrobial drug	Number (%) of resistant isolates		
	C. jejuni	***C. coli***	***C. jejuni* + *C. coli* (mixed)**
Ampicillin (AM)	2 (6.0%)	1 (16.6%)	6 (11.1%)
Ciprofloxacin (CI)	4 (12.1%)	1 (16.6%)	13 (24.0%)
Chloramphenicol (CL)	0	0	0
Erythromycin (EM)	0	0	0
Gentamicin (GM)	1 (3.0%)	1 (16.6%)	9 (16.6%)
Metronidazole (MZ)	7 (21.2%)	3 (50%)	19 (35.1%)
Tetracycline (TC)	0	1 (16.6%)	3 (5.5%)

Table 4. Multiple drug resistance pattern of *Campylobacter* isolates from backyard chickens

R-Pattern*	Number of isolates	Percent of isolates
***C. jejuni* (N=33)**		
AM, CIP	2	6
CIP, MZ	1	3
CIP, MZ, GM	1	3
Total	4	12
***C. coli* (N=6)**		
AM,MZ,TC,GM	1	16.6
CIP,MZ	1	16.6
Total	2	33.3
***C. jejuni* and *C. coli* (mixed infection (N=54)**		
MZ, GM	1	1.8
AM,MZ,GM	1	1.8
AM, MZ	2	3.7
CIP,MZ	2	3.7
CIP,MZ,GM	3	5.5
CIP,MZ,GM, TC	3	5.5
Total	12	22.2

AM, Ampicillin: CIP, Ciprofloxacin; GM, Gentamicin; MZ, Metronidazole; TC, Tetracycline; R, Resistance

4. DISCUSSION

In this study, 50.2% back yard chickens were positive for *Campylobacter.* These results are in agreement with previous researchers who reported high incidence of *Campylobacter* in commercially reared chickens [6,10,11,17] as well as in free range chickens [7,18-21]. Of the two species causing infections in humans (*C. coli and C. jejuni*), 90% are caused by *C. jejuni* [22]. The relative proportion of colonization by these two species varies in different host and geographical regions. *C. jejuni* is a major isolate from chicken meat in Ireland [3], France [21,23], Quebec -Canada [24], Finland [25], Denmark [26], Oxfordshire, UK [20], and Barbados [7]. However, *C. coli* has been reported as the dominant species in commercial layers and broiler chickens in Grenada [10,11], Thailand [27,28], and South Africa [29].

Using phenotypic criteria (hippurate test and nalidixic acid resistance), we found 70 *C. jejuni,* 21 *C. coli* and 2 non- typable isolates. When tested by gel-based PCR using genus-specific and *C. coli* and C. *jejuni* specific primers, 33 isolates were *C. jejuni*, 6 were *C. coli* and 54 were mixed infection with *C. coli* and *C. jejuni*. Although Nachamkin [14] advocates hippurate test as the most important phenotypic test to differentiate *C. jejuni* from *C. coli*, Ronner and Lindmark [30] and Ronner et al. [31] contradict the specificity of hippurate test. In the present study, hippurate test and nalidixic acid resistance tests failed to identify the specific species of the isolates.

Superiority of genotyping by PCR for species identification of *Campylobacter* has been proved and advocated by many authors [29,31,32]. Upon genotyping of isolates in the present study a majority (58.1%) was contaminated with mixed infection (*C. jejuni* and *C. coli*) whereas 35.5% and 6.5% cultures were pure *C. jejuni* and *C. coli, respectively*. Similar to our results of mixed infection, Rivoal et al. [18] reported a high percentage of mixed *C. jejuni* and *C. coli* infection in free range broiler farms in France. In a separate study in Belgium, Sabrina et al. [33] found overall mixed infection of 40.6% while *C. jejuni* was 46.9% and *C. coli* was 12.5% in free range broiler chickens. Reason for mixed infection especially in free range birds may be because of exposure to multiple sources of contamination [19,33]. As far as authors are aware, report on isolation of Campylobacters in free range chickens are scanty.

The microbial susceptibility test showed zero resistance for erythromycin and chloramphenicol. Erythromycin is the drug of choice for treatment of human campylobacteriosis, but our results show zero or minimum resistance to this drug as has been observed by others [10,34,35,36]. Another drug of choice for treatment of human campylobacteriosis is ciprofloxacin. Moderate resistance to ciprofloxacin was observed in our study as has been reported by previous researchers [10,34,35]. The higher rate of resistance to ciprofloxacin has been correlated with use of fluoroquinolones in other countries. In our study lower resistance was observed for tetracycline and ampicillin, which can be used for Campylobacter infections [35]. The resistance rate for tetracycline varied in different studies; Hariharan et al. [10] found 33.3% resistance for tetracycline against *C. jejuni* in commercial broilers. In a study in Canada, Guevremont et al. [37] reported 66% resistance for tetracycline in *C. jejuni* isolates from broilers. Absence of resistance for tetracycline in *C. jejuni* isolates in our study was similar to the observation of Frediani and Stephan [38].

Compared to other studies, in the present study, higher percentage of isolates showed resistance to metronidazole. Previous studies have reported variable resistance pattern for metronidazole. For example, Hariharan et al. [10] found 9.5% of the isolates resistant to metronidazole in Grenada while in a separate study in the same country, Rohini et al. [11] reported 34% resistance for metronidazole. Further, these authors [10,11] could not correlate the farm use of this drug to high percentage of resistance. As far as we are aware, there is paucity of literature on antimicrobial resistance pattern of *Campylobacter* isolates from backyard chickens and also on mixed infection with *Campylobacter* species. Although sensitivity tests should be done on pure cultures, our results on mixed culture are also presented to ensure a complete picture on what we found. A majority of reports show a correlation between microbial resistance in *Campylobacter* species and the use of drugs on commercial chicken flocks. Luangtongkum et al. [39] reported multiple drug resistance on chicken and turkey farms where antimicrobials were routinely used. Since the backyard chickens hardly get any medication, exact comparison of the susceptibility pattern with isolates of commercial chickens is not appropriate. A wider study on the antimicrobial pattern of the *Campylobacter* isolates from free range chickens is warranted.

Infection of humans with *Campylobacter*-contaminated meat and meat products has been well documented. Poultry is the major reservoir of thermophilic campylobacters. If the slaughter, handling and packing of meat from commercial poultry are done at modern slaughter facilities, hazard analysis critical control point (HACCP) can be well applied; HACCP has been found effective in reducing the meat contamination of poultry meat. The backyard chickens are usually slaughtered at home without implementing HACCP. This poses a risk to humans mainly through handling of contaminated meat of backyard chickens.

In conclusion, 50% of backyard chickens in Grenada were found positive for *Campylobacter* species. Persons keeping backyard chickens need be educated to observe HACCP while slaughtering and cooking of the meat from their flocks.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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