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Antimicrobial drug resistance as determined by the *E*-test in *Campylobacter jejuni*, *C. coli*, and *C. lari* isolates from the ceca of broiler and layer chickens in Grenada

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Abstract

One hundred and twenty five chickens from Grenada, consisting of 77 broilers and 48 layers were examined for carriage of thermophilic campylobacters in their ceca by culture. Seventy nine percent of chickens were positive for campylobacters, with an isolation rate of 93.5% for broilers and 56.3% for layers, the difference being significant. Sixty-four pure cultures comprising 39 *Campylobacter coli*, 21 *Campylobacter jejuni*, and 4 *Campyilobacter lari* isolates were tested for their resistance against 7 antibiotics using the *E*-test. None of the isolates were resistant to chloramphenicol and gentamicin. Resistance rates to other drugs were: ampicillin, 9.4%; ciprofloxacin, 12.5%; erythromycin, 3.1%; metronidazole, 9.4%, and tetracycline, 50% with MICs of $\geq$256 μg/mL for tetracycline. There were no significant differences in resistance rates between *C. coli* and *C. jejuni*. Multiple resistance to $\geq$2 drugs was seen in 15.6% of total isolates. All *C. lari* isolates were resistant to ciprofloxacin, and 3 of 4 isolates had multiple drug resistance. Overall, erythromycin, which is the drug of choice for treatment of *Campylobacter* infections in humans, is effective in vitro against 97% of chicken isolates in Grenada.

Keywords: *Campylobacter*; Grenada; Chickens; Antibiotic resistance

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Résumé

125 volailles de Grenade se composant de 77 poulets d'élevage et de 48 pondeuses ont été examinés en vue de la recherche par culture, de Campylobacter thermophile dans leur caecum.

70% des animaux ont été positifs avec un taux d'isolement de 93.5% chez les poulets d'élevage et 56.3% chez les pondeuse la différence étant significative.

64 cultures pures comprenants 39 *Campylobacter coli*, 21 *Campylobacter jejuni* et 4 *Campyilobacter lari* ont été testés pour leur résistance à 7 antibiotiques. Aucun des isolats s'est montré résistant au chloramphenicol et à la gentamicine. Pour les autres antibiotiques les taux de résistance ont été les suivants: ampicilline 9.4%, ciprofloxacine 12.5%, erythromycine 3.1%, metronidazole 9.4% et tetracycline 50%, avec un MICs > 256 μg/mL. Aucune différence de résistance n'a été observée entre *Campylobacter coli* et *Campylobacter jejuni*. Des résistances multiples à plus de deux antibiotiques ont été observées pour 15.6 isolats. Tous les *Campylobacter lari* étaient résistants à la ciprofloxacine et trois des quatre isolats présentaient des résistances multiples. L'erythromycine qui est le médicament de choix pour le traitement des infections à *Campylobacter* chez l'homme s'est montrée efficace in vitro dans 97% des isolats effectués chez les volailles de Grenade.

Mots clés : *Campylobacter* ; Grenade ; Poulet ; Résistance aux antibiotiques

1. Introduction

Campylobacteriosis is the most common food-borne bacterial disease in many developed and developing countries [1]. Epidemiological studies have revealed a strong association between *Campylobacter* infection in humans and handling and eating raw or undercooked poultry [2]. Contamination with even small amounts of cecal contents during processing can cause significant increase in the numbers of *Campylobacter* on eviscerated broiler carcasses [3]. Human disease can occur with an infective dose as low as 500 cells of *Campylobacter jejuni*. Extraintestinal infection, and late-onset complications such as Guillain–Barré syndrome (GBS), a paralytic disease condition, may occur in some patients [4]. Certain strains of *C. jejuni* may share antigens of peripheral nerve components, and antibodies that appear during recovery from *Campylobacter* enteritis may damage nerves resulting in GBS. Paralysis due to such *C. jejuni* strains has also been experimentally demonstrated in chickens [5].

Antimicrobial therapy, when indicated is often initiated before the results of susceptibility testing are available. Thus, a general knowledge of the expected susceptibility of *Campylobacter* species causing infections in a given geographic region is a prerequisite to initiate treatment with the most appropriate antimicrobial drug. Infections with *Campylobacter* species are most commonly treated with macrolides or fluoroquinolones [6]. It has been established that administration of antimicrobials to food producing animals can select for resistance among bacteria which are subsequently transferred to humans through food or animal contact [7]. Persons infected with ciprofloxacin resistant strains may have a longer duration of diarrhea, and more likely to be hospitalized, compared to patients with ciprofloxacin-susceptible infections [8]. In Grenada, no data are available on the presence and properties of campylobacters from animals, including poultry. Production of broiler chickens, and eggs is an economically significant undertaking in Grenada. Although fluoroquinolones are not being used in Grenada, it is known that a penicillin–streptomycin–multivitamin mixture is routinely used in broilers, and chlortetracycline is used for treatment of bacterial infection in chickens, including layers. It is important to monitor the antimicrobial drug resistance of the zoonotic campylobacters from chickens in various geographical areas for developing long-term measures aimed in protecting public health. This study was conducted for gathering information on resistance to 7 therapeutic drugs in thermophilic campylobacters, *C. jejuni*, *Campylobacter coli*, and *Campyilobacter lari* in broiler chickens and laying hens. Ten farms in Grenada were included in this study.

2. Materials and methods

One hundred and twenty five chickens from 10 farms were sampled. Of these 77 were broilers, and 48 were layers. Samples were collected on 8 different days during the period of August 2006 to January 2007. Samples from all birds slaughtered on a particular day in a farm were collected. The intestines, including ceca of the chickens were placed in sterile plastic bags and transported to the laboratory on ice for processing the same day. The ceca were aseptically opened and a loopful of contents was plated on Campylobacter-blood-free selective agar (CBF) with cefoperazone and amphotericin B supplements (Oxoid Ltd., Basingstoke, Hants, England). The plates were incubated under microaerophilic conditions employing Campy-Pak (Oxoid) at 42 °C for 48 h. Colonies were subcultured onto CBF plates after confirmation of typical morphology of campylobacters by Gram staining. The growths of pure cultures were transferred into 2% sterile skim milk in cryovials, and stored at −85 °C. Identification was based on key phenotypic properties recommended by Nachamkin [9]. Briefly, freshly grown cultures were subjected to 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 μg disk) and cephalothin (30 μg disk) (Oxoid) on 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 *E*-test (AB Biodisk, Solna, Sweden) for minimum inhibitory concentration was conducted according to the manufacturer's instructions on Mueller–Hinton agar (Remel) with 5% sheep blood. The inoculum was prepared from a fresh culture and bacterial suspensions were adjusted to MacFarland No. 1 turbidity standard (Remel) as per *E*-test manufacturer's instructions. After applying the *E*-test strip, the plates were incubated for 24 h at 42 °C in an anaerobic jar with a Campy-Pak (Oxoid). A *C. jejuni* strain susceptible to all 7 drugs, and giving reproducible MICs was used as a 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. Since there are no internationally accepted breakpoints for susceptibility or resistance for campylobacters, breakpoints established by the Clinical and Laboratory Standards Institute (formerly

National Committee for Clinical Laboratory Standards) for dilution test on aerobic bacteria have been found suitable by Luber et al. [10] and Guévremont et al. [11] for *C. jejuni/coli*. These breakpoints were used in the present study, and the MIC values used to classify a strain as resistant were: ampicillin and chloramphenicol, $\geq$32 µg/mL; ciprofloxacin, $\geq$4 µg/mL; erythromycin, $\geq$8 µg/mL; gentamicin and tetracycline, $\geq$16 µg/mL. The breakpoint for resistance to metronidazole was set at $\geq$16 µg/mL as recommended by Lorian [12].

Rates of isolation and antimicrobial resistance were statistically analyzed using the χ^2 test. For the calculations, Simple Interactive Statistical Analysis (SISA) on-line (http://home.clara.net/sisa/, dated 27 August, 2007) was used.

3. Results

Ninety-nine of 125 chickens (79.2%) were positive for campylobacters, with an isolation rate of 93.5% for broilers and 56.3% for layers. Compared to layers, the isolation rate was significantly higher for broilers (χ^2 = 22.7, d.f. = 1, $p < 0.001$). Of the 99 positive samples, only 64 isolates were obtained in pure and viable form (Table 1). All were positive for oxidase, catalase, and JCL agglutination reactions, and resistant to cephalothin. The most common species was *C. coli* with 39 isolates (60.9% of a total of 64), followed by *C. jejuni* with 21 isolates (32.8%). The remaining 4 isolates belonged to *C. lari* species.

Antimicrobial resistance for 64 *Campylobacter* isolates as determined by *E*-test against 7 drugs showed 0% resistance for chloramphenicol and gentamicin (Table 2), and only 3.1% resistance for erythromycin. Resistance to tetracycline was the highest, the rate being 50% for the total 64 isolates. The differences in rates for tetracycline resistance between *C. coli* and *C. jejuni* were statistically not significant (χ^2 = 2.6, d.f. = 1, $p > 0.05$). None of the tetracycline resistant isolates gave any zone of inhibition, the MICs being $\geq$256 µg/mL. All 4 isolates of *C. lari* were resistant to ciprofloxacin, while the resistance rates for *C. coli* and *C. jejuni* were <10%. All ciprofloxacin resistant isolates showed high levels of resistance, the MICs being $\geq$32 µg/mL. The resistance rates for ampicillin and metronidazole were relatively low for *C. jejuni* and *C. coli*, compared to that for the 4 *C. lari* isolates. Multiple drug resistance patterns (Table 3) were most common among *C. lari*, in comparison to *C. coli* and *C. jejuni*.

Table 1
Campylobacter isolates from ceca of 125 chickens from Grenada

	Number and % positive for campylobacters	
	Broilers (n = 77)	Layers (n = 48)
Positive for campylobacters	[a]72 (93.5%)	27 (56.3%)
Campylobacter coli[b]	32 (41.6%)	7 (14.6%)
Campylobacter jejuni[b]	16 (20.8%)	5 (10.4%)
Campylobacter lari[b]	4 (5.2%)	0 (0%)

[a] Based on growth on primary culture plates, and typical microscopic morphology. These include scanty growth with heavy yeast contamination and those which could not be re-isolated in pure form.

[b] Pure cultures that were identified to species level.

Table 2
Resistance of 64 *Campylobacter* isolates from chickens to 8 antibiotics as determined by *E*-test

Antibitotic	Number of resistant isolates and (*R*%)			Total
	C. coli (*n* = 39)	*C. jejuni* (*n* = 21)	*C. lari* (*n* = 4)	*n* = 64
Ampicillin	4 (10.3%)	0	2 (50%)	6 (9.4%)
Chloramphenicol	0	0	0	0 (0%)
Ciprofloxacin	2 (5.1%)	2 (9.5%)	4 (100%)	8 (12.5%)
Erythromycin	1 (2.6%)	1 (4.8%)	0	2 (3.1%)
Gentamicin	0	0	0	0
Metronidazole	(2.6%)	4 (19%)	1 (25%)	6 (9.4%)
Tetracycline	23 (59%)	7 (33.3%)	2 (50%)	32 (50%)

Table 3
Multiple drug resistance patterns of *Campylobacter* isolates from chickens

R-pattern[a]	Number of isolates	Percentage
C. coli (*n* = 39)		
Am, *Ery*, *Tet*	1	2.6
Cip, *Met*, *Tet*	1	2.6
Am, *Tet*	1	2.6
C. jejuni (*n* = 21)		
Ery, *Met*, *Tet*	1	4.8
Cip, *Tet*	1	4.8
Met, *Tet*	2	9.5
C. lari (*n* = 4)		
Amp, *Chl*, *Cip*, *Met*, *Tet*	1	25
Amp, *Cip*	1	25
Cip, *Tet*	1	25
Total (*n* = 64)	10	15.6

[a] *Amp*, ampicillin; *Chl*, chloramphenicol; *Cip*, ciprofloxacin; *Ery*, erythromycin; *Met*, metronidazole; *Tet*, tetracycline.

4. Discussion

Seventy nine percent of 125 chickens sampled were positive for campylobacters, which is in agreement with documented infection rates of commercially reared chickens [13]. However, *C. coli* was isolated on subculture significantly at a higher rate than *C. jejuni* in the present study, which contradicts published information that poultry are colonized primarily with *C. jejuni*, less often with *C. coli* and rarely other species. However, the proportion may vary from country to country, and *C. jejuni* could be 65% of total campylobacter isolates, as in the Netherlands, or higher as in UK [14]. In a study in France [15], the percentage of isolation of *C. coli* was significantly higher when birds received β-lactam antibiotics. The broilers in Grenada are routinely treated with a 'growth booster' mixture containing penicillin, and this could be one of the reasons for the higher isolation rate for *C. coli*. A possibility also exists that up to 10% of chicken isolates classified as *C. coli* on the basis of negative hippurate test results may be identified as *C. jejuni*, if genotyping is done for confirmation [16]. Nevertheless hippurate test is the most important phenotypic test to differentiate *C. jejuni* from *C. coli* [9].

C. lari, a nalidixic acid-resistant species, commonly found in seagulls (genus *Larus*) is known to produce acute diarrheal illness and/or bacteremia, which may be fatal in humans [17,18]. Isolation rate for *C. lari* in the present study was 6.3%, and all were from broilers. Published data on isolation rates of *C. lari* from chickens is lacking. However, in a survey of campylobacters in broilers slaughtered in Denmark, only 1% belonged to *C. lari* species [19].

For susceptibility testing of campylobacters, a number of different diffusion and dilution methods are used in various laboratories. However, the *E*-test has given comparable results with broth dilution and agar dilution methods [10,20]. Although antimicrobial therapy is not required for the great majority of human patients with *Campylobacter* enteritis, there are several indications for use of antibiotics. These include acute illness with no improvement, systemic infection and immunosuppression. The most commonly used drugs are erythromycin, which is the first choice, and fluoroquinolones, especially ciprofloxacin. Overall resistance of campylobacter isolates from chickens to erythromycin was only 3.1% in this study, which is encouraging. Resistance to ciprofloxacin was 12.5% in this study, while a study of campylobacters in broiler chickens in Trinidad showed a rate of 86.6%, the reason being the routine use of quinolones in poultry in Trinidad and Tobago. Resistance to quinolones is 100% in *C. lari* because quinolone resistance is intrinsic in this species [21]. Fluoroquinolone*s* are not being used in poultry in Grenada. Smith et al. [7] summarized country-specific data showing evidence, suggesting that fluoroquinolone use in poultry largely explains the increasing fluoroquinolone resistance among *Campylobacter* isolates. The emergence and increase of drug resistant strains in broilers has been closely followed by an emergence and subsequent increase in resistance among isolates causing infections in humans [22,23]. Aminoglycosides such as gentamicin are generally used for serious systemic infections due to campylobacters [4]. All isolates including *C. lari* in this study were susceptible to gentamicin and chloramphenicol. Likewise, in a study on antimicrobial resistance among *Campylobacter* strains in the United States by the National Antimicrobial Resistance Monitoring System (NARMS) during 1999, Gupta et al. [8] found none of the campylobacter isolates from retail chicken resistant to gentamicin and chloramphenicol. Resistance to erythromycin was low as in the present study. Erythromycin resistance in *C. jejuni* is of concern, and resistant isolates have appeared in poultry in Ontario, Canada [24]. However, resistance to ciprofloxacin among *C. jejuni* isolates in the NARMS study in US [8] on retail chicken isolates was 24%, as opposed to 9.5% in cecal isolates from chickens in the present study. The NARMS study [8] gave a rate of 46% tetracycline resistance among human *C. jejuni* isolates, compared to 33.3% rate among chicken *C. jejuni* isolates in the present study. Chlortetracycline is used to treat chickens against bacterial diseases in Grenada. In a study of *C. jejuni* isolates from chicken ceca in Canada, 66% showed resistance to tetracycline, the reason being common use of tetracyclines for prophylaxis and growth promotion in poultry [11]. In contrast, a Swiss study [20] on 195 *C. jejuni* strains isolated from poultry carcasses, showed 0% resistance to tetracycline. In a study in France [25], a quarter of *C. jejuni* isolates were resistant to tetracycline, while the rate was 83% for *C. coli*. In the present study, although the rate of resistance to tetracycline in *C. coli* was higher than that of *C. jejuni*, the difference was statistically not significant. The results of the present study also differed from the French study [25] with regard to the resistance rates for erythromycin, which was much higher among *C. coli* in the latter. Frediani-Wolf and Stephan [20] found significantly higher percentages of resistance among isolates from normal production flocks compared to free-range flocks. The overall resistance among 64 isolates to metronidazole in the present study was only 9.5%, and for *C. jejuni*, the rate being 19%. In contrast, Stanley and Jones [26] found >90% of *C. jejuni* isolates from avian sources including broiler chickens resistant to metronidazole, while <20% of isolates from ruminants were resistant. A more recent study in Denmark [27] showed higher metronidazole resistance in *C. jejuni* from turkey meat compared to broiler meat. A study on 14 *C. jejuni* isolates from double-crested cormorant chicks in Canada [28], none was showed resistance to metronidazole. Susceptibility of *C. jejuni* strains to metronidazole, a drug primarily used to treat anaerobic infections in animals and humans, is highly variable [29]. The results of the present study suggest that metronidazole resistance may not be a useful epidemiological marker for avian strains.

In conclusion, results of the present study show that thermophilic *Campylobacter* species colonize broilers more than layers in Grenada, and that 97% of chicken isolates are susceptible to erythromycin, a drug recommended for treatment of human campylobacteriosis.

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