

Antimicrobial Resistance Profiles of *Campylobacter jejuni* Isolates from Wild Birds in Sweden†

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In order to determine the occurrence and frequency of resistant strains of the bacterium *Campylobacter jejuni* and to establish baseline MICs in isolates from an environmental reservoir, the resistance profiles of 10 antimicrobial substances were determined for 137 *C. jejuni* isolates from wild birds in Sweden. Observed MICs were generally low, with only low to moderate incidence of resistance to the tested compounds. One isolate, however, was resistant to nalidixic acid and ciprofloxacin, indicating that quinolone-resistant genotypes of *C. jejuni* have the potential to spread to wild bird hosts.

Currently, there is an intense debate on the use and misuse of antimicrobial agents in the raising of livestock for food and their sometimes imprudent prescription for therapeutic use in humans (2, 12). From the farm perspective, animal health and farm economics is balanced against the associated risk of bacterial pathogens acquiring antimicrobial resistance (7, 19). As laws and control measures differ between countries, there are often large variations between nations in their total and per capita use of antimicrobial agents in both human and veterinary medicine, with a general trend towards more unrestricted use in developing countries.

The enteropathogens *Campylobacter jejuni* and *Salmonella* spp. represent an important public health problem. Domestic animals constitute a significant reservoir for these zoonotic agents that are typically food borne (17, 21). Resistance acquired by these bacteria in response to antimicrobial selection pressures in livestock production could severely hamper the treatment of human infections, especially when the same class of antimicrobial agents is used in the two systems (2, 26). In a worst-case scenario, with multiresistant bacteria, there would be few, if any, antimicrobial compounds available for clinical use.

One important question specifically addressed in the present study is whether resistant bacteria are directly or indirectly “leaking” out from humanmade systems into the environment. The study focuses on *C. jejuni*, one of the leading causes of acute bacterial gastroenteritis in humans (11), and analyzes the antimicrobial resistance profiles of *C. jejuni* isolates obtained from a large collection of wild birds. Important risk factors for acquiring *C. jejuni* infection include consumption of undercooked poultry meat, cross-contamination from poultry meat to other food products, and drinking inadequately pasteurized milk or untreated drinking water (3, 11, 23). There are, how-

ever, good reasons to believe that less well-known sources may be important in the epidemiology of the bacterium, as many outbreaks and sporadic cases are left unresolved (16, 20). Apart from the well-known poultry reservoir, *C. jejuni* occurs naturally in both domesticated and wild mammals and birds (27), and it has been isolated from surface and groundwater (16). Taken together, these elements set the stage for potential transfer of resistant strains in and out of food production systems.

In the present study, we determined the susceptibility of 137 *C. jejuni* strains isolated from free-flying healthy birds. The tested strains were chosen to represent three different groups of bird hosts: thrushes, shorebirds, and raptors, all previously known to harbor significant levels of *C. jejuni* (5, 27).

MATERIALS AND METHODS

Origin of strains. The *C. jejuni* strains investigated in this study came from ongoing studies of the occurrence and distribution of *Campylobacter* spp. in different free-flying bird species in Sweden. In this project, birds were trapped and sampled at Ottenby Bird Observatory (56° 12'N, 16° 24'E) when they stopped over in the area during their northbound spring or southbound autumn migration. For details on trapping, sampling, and primary isolation procedures, see previously published descriptions (6, 27). One-hundred thirty-nine strains, presumptively identified as *C. jejuni* based on morphology and limited phenotypic testing (catalase, oxidase, and hippurate hydrolysis), were chosen from a larger collection of strains in order to achieve representatives from three different groups of wild birds: thrushes (family *Muscicapidae*, genus *Turdus*), raptors (families *Strigidae* and *Accipitridae*, genera *Asio* and *Accipiter*), and shorebirds (family *Scolopacidae*, genera *Calidris*, *Limicola*, *Tringa*) (Table 1). The presumptive bacterial species identity was later validated using molecular methods. All isolates were first tested in a multiplex PCR method that gives separate species-specific amplifications of *C. jejuni* and *C. coli* genes (25). PCR conditions and reactions were performed as described in the original study (25). Template DNA for the PCRs consisted either of purified chromosomal DNA (92 isolates; Puregene DNA Isolation Kit; Genra Systems, Minneapolis) or extracted DNA from 48-h-old subcultures grown on heart infusion (HI) agar (Difco; product no. 244400), supplemented with 5% sheep blood, using a 20% Chelex-100 slurry (Bio-Rad; product no. 142-2842), as described previously (9). Clear positive amplicons specific for *C. jejuni* were observed for all but four tested isolates. The remaining isolates were subjected to a previously described combined PCR and restriction fragment analysis method (10), where three isolates gave *C. jejuni*-specific restriction patterns and the last *C. coli*-specific pattern (this isolate was excluded from the study).

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TABLE 1. Number of *Campylobacter jejuni* isolates from different host species placed into ecological groups

Group	Host species	n
Thrushes	Blackbird	41
	<i>Turdus merula</i>	
	Song thrush	39
	<i>Turdus philomelos</i>	
	Redwing	11
	<i>Turdus iliacus</i>	
	Fieldfare	2
	<i>Turdus pilaris</i>	
	Mistle thrush	1
	<i>Turdus viscivorus</i>	
Raptors	Long-eared owl	8
	<i>Asio otus</i>	
	Sparrowhawk	1
	<i>Accipiter nisus</i>	
Shorebirds	Dunlin	31
	<i>Calidris alpina</i>	
	Broad-billed sandpiper	1
	<i>Limicola falcinellus</i>	
	Curlew sandpiper	1
	<i>Calidris ferruginea</i>	
	Little stint	2
	<i>Calidris minuta</i>	
	Wood sandpiper	1
	<i>Tringa glareola</i>	
Total		139

Susceptibility tests. Susceptibility was tested quantitatively with an adjusted broth microdilution test according to NCCLS guideline M31-A2. For broth microdilution, microtiter trays were used with dehydrated dilution ranges of custom-made panels of antimicrobial agents (Trek Diagnostic Systems, United Kingdom). The agents included in the panels are presented in Table 2. After inoculation of the microtiter trays with 50 µl of a 200-fold-diluted 0.5 McFarland suspension in Mueller Hinton-II broth (MH-II broth; Lab M; product no. Lab 39, supplemented with 0.2 IU/ml thymidine phosphorylase; Sigma; product no. T7006) of a 48-h-old pure culture on blood agar, the trays were incubated microaerobically in a shaking incubator at 37°C for 24 h (80 to 100 rpm). *C. jejuni* ATCC 33560 was used as the control strain. All isolates from thrushes and one from an owl failed to grow sufficiently in MH-II broth, and for these strains heart infusion (HI) broth (Difco; product no. 238400) was used instead. The MICs were defined as the lowest concentrations in the wells where no visual growth could be observed.

RESULTS

Of the 139 bird isolates, 94 failed to grow sufficiently in MH-II broth. For these isolates HI broth was used in the microdilution tests. One isolate failed to grow also in the HI broth and, consequentially, its resistance profile could not be determined. Another isolate was identified as *C. coli* and was omitted from further analyses. The MICs for the control strain *C. jejuni* ATCC 33560 to ciprofloxacin, nalidixic acid, and erythromycin were always within tentative quality control (QC) ranges for the agar dilution method (NCCLS document M31-A2), irrespective of the broth used. The MIC of the control strain to doxycycline was only once one dilution step below the QC ranges, and the MIC to gentamicin was four out of six times one dilution step below the QC ranges. For the other antimicrobial agents, QC ranges did not exist yet, so the results were interpreted with more care. For *C. jejuni* ATCC 33560 the MIC to amoxicillin varied between 4 and 8 µg/ml, to chlor-

amphenicol the MIC varied between ≤2 and 8 µg/ml, to metronidazole the MIC varied between ≤0,5 and 1 µg/ml, to neomycin the MIC varied between ≤0,5 and 2 µg/ml, and to streptomycin the MIC varied between ≤1 and 2 µg/ml, irrespective of the broth used.

All isolates were found to be susceptible to chloramphenicol, erythromycin, gentamicin, neomycin, and streptomycin (Table 2). Low resistance prevalences (0.7 to 3.6%) were observed for four compounds: amoxicillin, ciprofloxacin, doxycycline, and nalidixic acid, whereas a moderate resistance prevalence (14.6%) was found to metronidazole (Table 2).

When all substances were considered, two of the bird species, long-eared owl and blackbird, more often carried resistant *C. jejuni* isolates than other species (Table 3). This was particularly evident for metronidazole. Notably, the eight isolates from long-eared owls showed resistance to four of the tested compounds, and one of these was simultaneously resistant to nalidixic acid and ciprofloxacin.

DISCUSSION

The use of antimicrobial agents in the rearing of poultry and other food-producing animals is perceived by many as a strong threat to public health, as bacteria in these systems, including pathogenic ones, are under strong selection for acquiring resistance to antimicrobial compounds. The threat can be divided into two parts: first, resistant zoonotic bacteria that survive all production steps could end up in the food consumed by humans; second, selection for anti-antimicrobial genes could spread these alleles within clonal complexes of bacteria or be transferred horizontally to other bacterial species. In the latter case, resistance genes selected for in a harmless commensal bacterium could jump to a pathogenic species, where human or animal health considerations are much greater. Resistant genotypes could spread from farms into the environment by a number of ways, but the important question to ask is whether these genotypes can persist in, and further spread to, any environmental reservoirs such as wild birds. If so, a feed-back loop from one farm through the environmental reservoir to the next could be possible.

Overall, the incidence of resistance to antimicrobial agents was low for the investigated wild bird isolates. There are a number of published studies in the human and veterinary literature on the degree of acquired resistance to antimicrobial compounds; many that report considerably higher incidences of resistance than those seen in this study (1). For instance, data obtained in the same lab as in this study on the occurrence of resistant isolates of *C. jejuni* in Dutch humans (1998 to 1999) and Dutch poultry (2000 to 2003) reported that 7.8% and 24.3% of investigated strains were resistant to amoxicillin, 23.4% and 36.3% to ciprofloxacin, and 16.9% and 27.1% to doxycycline, respectively (15). The wild birds in this study were sampled during their annual migrations between breeding and wintering areas and vice versa. Thus, the *C. jejuni* strains may have colonized the birds in areas far from the sampling site. However, values from Swedish broiler chickens (4) were rather similar to those observed in the wild bird isolates, with 7.1% of isolates resistant to ampicillin, 0.8% to enrofloxacin, and 0.8% to tetracycline (2001 to 2002; n = 127).

The highest resistance prevalences were seen in isolates

TABLE 2. MIC values to different antimicrobial compounds in 137 *Campylobacter jejuni* isolates from wild shorebirds (A), thrushes (B), and raptors (C)^a

Drug	Bird	Amt of drug (µg/ml)														n	Breakpoint for resistance (µg/ml)	Resistance
		0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024			
Amoxicillin	All		1	2	2	23	31	45	28	5						137	>16	3.6
	A						1	8	26							35		0.0
	B		1	2	2	22	28	33	2	3						93		3.2
	C					1	2	4		2						9		22.2
Chloramphenicol	All					101	33	3							137	>16	0.0	
	A					24	11								35		0.0	
	B					68	22	3							93		0.0	
	C					9									9		0.0	
Ciprofloxacin	All	58	48	21	9			1							137	>2	0.7	
	A	12	22	1											35		0.0	
	B	38	26	20	9										93		0.0	
	C	8						1							9		11.1	
Doxycycline	All	101	25	7	2	1				1					137	>4	0.7	
	A	34								1					35		2.9	
	B	59	25	7	2										93		0.0	
	C	8				1									9		0.0	
Erythromycin	All			15	49	56	10	7							137	>16	0.0	
	A			12	15	6	2								35		0.0	
	B			1	28	49	8	7							93		0.0	
	C			2	6	1									9		0.0	
Gentamicin	All		70	67											137	>8	0.0	
	A		35												35		0.0	
	B		26	67											93		0.0	
	C		9												9		0.0	
Metronidazole	All			15	55	38	9	5	8	5	1	1			137	>4	14.6	
	A			1	24	8			1	1					35		5.7	
	B			12	28	30	9	3	7	3		1			93		15.1	
	C			2	3				2		1	1			9		44.4	
Nalidixic acid	All				1	2	45	47	38	3		1			137	>32	0.7	
	A						8	22	5						35		0.0	
	B						32	25	33	3					93		0.0	
	C				1	2	5					1			9		11.1	
Neomycin	All			63	66	7	1								137	>8	0.0	
	A			34	1										35		0.0	
	B			21	64	7	1								93		0.0	
	C			8	1										9		0.0	
Streptomycin	All				132	5									137	>8	0.0	
	A				34	1									35		0.0	
	B				89	4									93		0.0	
	C				9										9		0.0	

^a MICs are in micrograms per milliliter. Also given are MIC breakpoint values and the corresponding incidence of resistance (in percentages).

from the raptor group, which consisted mainly of long-eared owls. From one of these owls, an isolate with resistance to both nalidixic acid and ciprofloxacin was retrieved. The resistance to fluoroquinolones is caused by a point mutation in the *gyrA* gene (18) and has been shown to occur rapidly both in vitro and in vivo in the presence of this class of antimicrobial compounds (13, 18, 24). The few studies that have examined ge-

netic similarities of *C. jejuni* strains isolated from wild birds to those of chickens and humans have shown that wild bird strains generally are different from isolates from other sources, but that similar genetic fingerprints sometimes occur (5, 6). The finding of a quinolone-resistant *C. jejuni* isolate could thus represent a resistant phenotype that has originated within another reservoir. Long-eared owls are essentially nocturnal and

TABLE 3. Seasonal distribution of isolates resistant to tested antimicrobial agents (numbers of the isolates are placed in brackets)

Host species	Amoxicillin	Ciprofloxacin	Doxycycline	Metronidazole	Nalidixic acid
<i>Accipiter nisus</i>	Sep (1)				
<i>Asio otus</i>	Nov (1)	Oct (1)			
<i>Turdus merula</i>				Apr (2), Oct (1), Nov (1)	Oct (1)
<i>Turdus philomelos</i>	Apr (2)			Apr (5), Oct (2), Nov (2)	
<i>Turdus viscivorus</i>	Mar (1)			Oct (2)	
<i>Turdus iliacus</i>				Apr (1)	
<i>Calidris alpina</i>			Jul (1)	Jul (2)	
<i>Tringa glareola</i>					
<i>Limicola falcinellus</i>					
Total	5	1	1	20	1

feed mainly on small rodents (especially voles *Microtinae*, and mice *Murinae*), but they also include passerine birds in their diet (ca. 2 to 17% of identified remains from pellets [8]).

Elevated MIC values were also observed among wild bird isolates for amoxicillin, doxycycline, and metronidazole. For amoxicillin, however, all the five isolates in question produced MIC values that were only one dilution step above the breakpoint of 16 µg/ml (Table 2). The varying MICs of 4 to 8 µg/ml for the internal standard, *C. jejuni* ATCC 33560, to this compound indicate that the methodology may have been suboptimal. An allowance for a methodology error of one dilution step would place all isolates scored here as resistant below the breakpoint. The single isolate that showed resistance to doxycycline (Table 2), obtained from a Dunlin (*Calidris alpina*), grew in 4-dilution-steps higher concentration than did the isolates with the second highest MIC. Therefore, the elevated MIC in this isolate is more likely to reflect a true resistance. A total of 14.5% of all isolates had MIC values for metronidazole that exceeded the breakpoint. There was also a tendency to a bimodal MIC value distribution for this substance. Metronidazole-resistant isolates were found in all three bird groups but were more frequent among raptors and thrushes (Table 2). The mechanisms behind the resistance to metronidazole in *Campylobacter* spp. are not yet fully understood. Reduction of the 5-nitro group of the imidazole ring to nitro-radical anions responsible for the species-specific effects on DNA have been described in anaerobes and is probably also responsible for the activity in *Campylobacter* species. In the related bacterium *Helicobacter pylori*, resistance to metronidazole can result from the loss of activity of an oxygen-independent NADPH nitroreductase due to mutations in the *rdxA* gene. However, the existence of this pathway in *Campylobacter* spp. has not been validated (14). The observation that metronidazole resistance in *C. jejuni* is associated with strains isolated from birds was described previously by Stanley and Jones (22) and is confirmed by the results of the present study. Selection for metronidazole resistance by the use of dimetridazole derivatives in birds was considered highly unlikely, and a host phenotype relationship was suggested (22).

The present study provides valuable baseline data on MIC values in *C. jejuni* isolates from three groups of wild birds sampled in Sweden. Most of the host species studied here are not normally found close to human settlements, and the results could have been different if we had studied bird species that were more associated with human activities. Nevertheless, we found one bacterial isolate with resistance to quinolones, indicating that resistant genotypes can leak out into the wild bird reservoir.

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