MICROBIAL SUSCEPTIBILITIES AND RESISTANCES TO *CAMPYLOBACTER JEJUNI* ISOLATESFROM CROW FECAL MATTER IN WASHINGTON STATE

Neha Chhabra

ABSTRACT: This research investigates the antimicrobial resistance profile of Campylobacter jejuni (C. jejuni) isolates obtained from crows within Bothell, Everett, Mercer Island and Factoria, Washington. While campylobacteriosis is a self-limiting disease, severe or prolonged cases of campylobacteriosis need treatment with antibiotics. However, antibiotic resistance (AR) can develop. Antibiotic resistance is a growing public health issue because resistance to some antibiotics defeats the purpose of having antibiotics for treatment. This study seeks to scrutinize the antimicrobial susceptibilities and resistances of forty Campylobacter jejuni isolates obtained from crow feces from different sites around Washington State. Crows were used during this study because they can serve as environmental indicators of the antibiotic usage of the places they visit during their daytime scavenging activities. Kirby-Bauer method was used with eleven antibiotics, including tetracycline, ampicillin, ampicillin-calvulanic acid, ciprofloxacin, chloramphenicol, streptomycin, nalidixic acid, azithromycin, erythromycin, gentamicin, and clindamycin. The C. jejuni isolates showed maximum resistance to ciprofloxacin (34.21%) and to tetracycline (31.5%). Sixteen total isolates showed multiple drug resistance (resistance to three or more antibiotics). Two isolates, F18 and F19-2 obtained from the Bothell wetlands showed resistance to 6 antibiotics, including intermediate susceptibility. These results show that crows are capable of spreading antibiotic resistance through the bacteria C. jejuni.

Keywords: Campylobacter jejuni, antibiotic resistance

Introduction

The fundamental goal of this study was to determine the antimicrobial resistance profiles for the *Campylobacter jejuni* (*C. jejuni)* isolates within Northwestern Washington State. Research performed in the United States, Kolkata, India and China have observed results for their respective countries on antibiotic resistance in *C. jejuni.* According to the World Health Organization, the *Campylobacter* species are extensively dispersed in most warm-blooded animals and prevalent in food animals like poultry, cattle, pigs, sheep, ostriches, and in pets, including cats and dogs. The bacteria have also been found in shellfish (WHO, 2018). Raw or contaminated milk, undercooked meat or meat products, contaminated water or ice are all routes of transmission from animals to humans and thus *Campylobacter* infections are usually foodborne. Antimicrobials used in food-producing animals have been associated with the development of resistance in both *C. jejuni* and *C. coli* (McDermott et al., 2002). *C. jejuni* is an important organism to study because it is the most commonly reported bacterial cause of gastrointestinal disease in the United States. A result of having a *Campylobacter* infection could be Guillain-Barré syndrome (GBS), a disorder resulting in acute neuromuscular paralysis, and 40% of patients with the syndrome have evidence of a recent *Campylobacter* infection (Altekruse,

1999, para 4). Diseases or infections that have antibiotic resistant bacteria are more difficult to treat and have a heavier impact on individuals. Therefore, resistant infections can become an issue. They feed on animal and human waste among other things, and thus obtain and carry the bacteria from these substances. The aim of this research is to determine antibiotic sensitivity and resistance patterns of *Campylobacter jejuni*. Crow *C. jejuni* isolates were investigated because they can be important indicators of the AR of that environment.

Methods & Materials

Rationale and Application of Techniques: Protocol

Growing Isolated Strains

50 fecal samples of crows from Seattle, Washington, USA, were obtained during 2014-2015. At a singular time, fresh fecal samples from individual birds (3-8 samples) were streaked on campy CVA blood agar for the selective isolation of *Campylobacter*. Campy CVA blood agar contains peptamin that can provide carbon, sulfur, and nitrogenous compounds required for growth. Yeast extract provides B vitamins to the medium, and dextrose is incorporated as an energy source. Sheep blood supplements the medium with X-factor and other growth factor requirements (Hardy Diagnostic, 1996). Details of the collection can be found in (Sen et al., 2018, pp. 1-13). Isolates were stored as glycerol stocks at -80° C. For the antibiotic resistance studies, the stocks were streaked out on CVA blood agar. All methods incorporated aseptic techniques and procedures that maintained the sterility of all experimental materials. The plates were incubated at 37°C under microaerophilic conditions (5% O2, 10% CO2, and 85% N2) by using a CampyGen pouch (Oxoid Limited), with first observation after a 36-48-hour incubation period. Individual grown colonies of *C. jejuni* were collected using proper sterilization technique with flaming an inoculating loop and spinning it between fingers into ~1mL of Trypticase Soy Yeast (TSY) broth. The turbidity of the suspension was measured using a spectrophotometer, in which a control TSY broth maintained the 'blank' status, and suspensions having an optical density at 600 nm between 0.060-0.090 was used.

On Müller-Hinton (MH) Blood Agar Plates, 100µL of the suspension was streaked using a turntable hockey stick in fast back-and-forth motion while spinning the plate. The plates were allowed to air dry for 10-15 minutes. For each isolate, two MH Blood Agar plates were used to calculate the antibiotic resistance. Using forceps and aseptic techniques, a total of 11 antibiotics were placed on two separate plates: five antibiotic disks were placed on one plate for an isolate, and six antibiotics were placed on the other plate for the same isolate. Antibiotic disks were placed 15 mm from the edge of the plate and 20 mm from each other, except for the sixth one, which was placed in the center (*Figure 1*). Up to four plates were placed in a plate-sealing bag with a dampened paper towel and CampyGen pouch (Hardy Diagnostics, 1996), a self-contained gas generating system used in closed environmental chambers to rapidly generate microaerophilic atmospheres, which are essential for the isolation and growth of *Campylobacter*. After a 36-48-hour incubation period under microaerophilic conditions as described above, antibiotic disks were read. In this study, the American Type Culture Collection *C. jejuni* strain, ATCC 33560 was used as a quality control organism.

Analysis of AR Disks

After incubation, the plates were inspected for zones of growth inhibition around the colonies. This method identifies bacterial strains that produce inhibitors in the agar. It is a circular area around the spot of the antibiotic in which the bacterial colonies do not grow. In table 1, zone diameters are observed from *Enterobacteriaceae* values. Using an electronic caliper, diameters were measured from one end of the growth to the other. If results were obscure or growth was blended into the next antibiotic, radii of the zone

of inhibition were measured and then doubled to get the complete zone of inhibition. The zone diameters were checked according to Clinical and Laboratory Standards Institute guidelines. The CLSI clinical breakpoints for an antibiotic towards *Enterobacteriaceae* were used to assign isolates sensitive, reduced susceptibility or resistant status (Table 1).

Creating DNA

Several representative isolated colonies were obtained using a sterilized inoculating loop and suspended into 100µL of PrepMan Ultra Sample Preparation Reagent (Life Technologies). The suspension was heated in a heating block at 95°C, to break the cells open to release the DNA, for ten minutes. The tubes were cooled to room temperature or 50°C for about a minute, and then placed in a centrifuge to precipitate the cell debris into a pellet. The supernatant containing the DNA was saved for multi-locus sequencing typing and other genetic studies.

Storing in TSY + glycerol

Isolates from the initial incubation period were used in creating bacterial glycerol stock. A 30:70 ratio of 50% glycerol and TSY broth was mixed to use as a freezing medium to store *C. jejuni* isolates. Using a sterile cotton tip applicator, isolates were wiped and collected from the plate, then suspended into 400µL TSY + glycerol medium. The goal of the stock is to produce a thick slurry. This was immediately put on ice and within 30 minutes stored at -80°C.

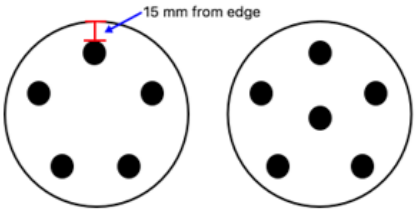
Results and Discussion

Table 2 shows 11 antibiotics used for susceptibility testing of *Campylobacter jejuni* isolates. Resistant phenotypes were determined highest for ciprofloxacin, and for tetracycline in which 13 out of 40 isolates, (34.21%) and 12 out of 40 isolates (31.5%) were resistant, respectively. Thirty-six out of forty *C. jejuni* isolates were susceptible to gentamicin (92.30%) and thus gentamicin seems to be the most effective against these isolates. F28 and F43 showed similar AR profiles in that they were susceptible to all antibiotics except for gentamicin against which F28 was resistant, and F48 had reduced susceptibility. Both these isolates belonged to the same cluster by *fla*SVR analysis, although they were collected on 2 separate dates. Being sister taxa, B56 and B8 show similar results in that they were both resistant towards ciprofloxacin, but only B56 showed resistance towards nalidixic acid while F81 showed susceptibility. Although being of the same common ancestry, and from the same collection dates, F21.2 and F23-2 did not show similar results in that some antibiotics were completely sensitive whereas others were completely resistant (Figure 2, Table 2). For example, F21.2 showed resistance or reduced susceptibility to ciprofloxacin, nalidixic acid, and streptomycin whereas F23-2 showed only susceptibility. F61 and F62 also showed

Table 1. Zone diameters according to Clinical and Laboratory Standards Institute guidelines (CLSI)

		Resistant	Reduced Susceptibility	Sensitive
Ampicillin	AM-10	<=13	14-16	>=17
Ampicillin-Clavulanic Acid	AMC-30	<=13	14-17	>=18
Azithromycin	AZM-15	<=13	14-17	>=18
Chloramphenicol	C-30	<=12	13-17	>=18
Ciprofloxacin	CIP-5	<=15	16-20	>=18
Clindamycin	CC-2	<=14	15-16	>=17
Erythromycin	E-15	<=13	14-22	>=23
Gentamicin	GM-10	<=16	17-19	>=20
Naladixic Acid	NA-30	<=13	14-18	>=19
Streptomycin	S-10	<=11	12-14	>=15
Tetracycline	TE-30	<=14	15-18	>=19

Figure 1. Placement of 11 antibiotics on 2 separate MH Blood Agar Plates for a singular isolate



significantly different results although both were collected on the same dates from Factoria, WA. They were similar in that both showed reduced sensitivity to streptomycin, and clindamycin but different resistant values to ampicillin, nalidixic acid, ciprofloxacin, and tetracycline. Isolates F83 and B32 showed exact same results in that all antibiotics proved to be susceptible. B42 and B44 were different in their AR profile although they were collected on the same day but from different fecal samples. There is a chance that F42 growth may not have been *Campylobacter*

Figure 2. Phylogenetic tree indicating ancestry (clustering together) of isolates from crow fecal samples. "KOL" for Kolkata samples. "B" for UW Bothell (Reproduced from Sen, et al. Appl Env. Microbiol: (2018): 84.6 e01893-17. Figure 6).

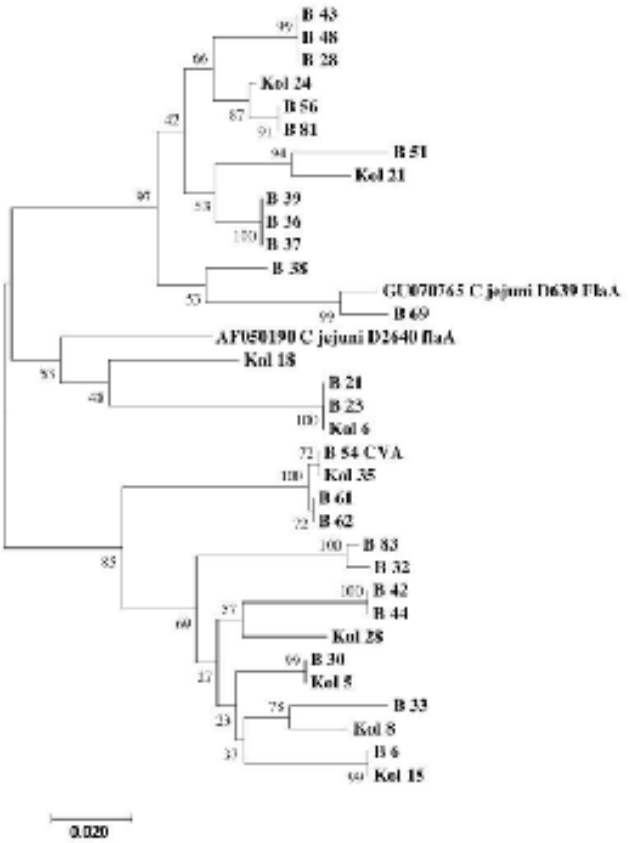


Table 2. Antibiotic resistance values of Campylobacter jejuni, measured in millimeters using an electronic caliper. The last column indicates the total number of antibiotics that an isolate was resistant to. The ATCC 33560, control strain, was sensitive to all antibiotics. ND indicates not determined.

Antibiotic	Tetracycline TE30	Erythromycin E15	gentamycin GM10	clindamycin CC2	azithromycin AZM15	ciprofloxacin CIP5	chloramphenicol C30	Nalidixic acid NA30	Streptomycin S10	Amox. Clavulinic Acid AMC30	Ampicillin AM10	Resistant to # of antibiotics
P18	21.9	10.32	16.9	R	13.97	32.6	ND	18.45	16.44	16.18	11.45	5
P19.2 (10/1/18)	26.29	R	29.79	R	R	14.81	11.47	22.51	25.51	23.17	13.27	6
P20.2	R	26.26	29.23	26.19	24.24	39.74	38.48	22.14	27.86	40.28	30.34	1
P21.2	11.85	11.88	14.66	28.18	38.19	R	30.99	R	11.48	34.95	31.6	4
P22.2	ND	control	34.86	23.94	ND	37.38	32.78	25.1	38.33	43.32	39.82	5
P23.2	R	ND	39.76	32.9	45.18	32.24	42.4	31.71	24.02	45.06	33.56	1
P28	21.84	29.15	R	25.8	35.56	32.96	35	26.84	31.7	39.16	ND	1
P29	3.03	ND	31.76	26.37	36.45	33.92	33.67	37.47	7.03	40.48	46.56	2
P30	34.94	31.66	36.06	30.94	30.82	40.62	32.92	24.57	26.67	31.39	35.14	1
P31	S	S	S	S	S	S	S	S	S	S	S	0
P31.1	R	24.24	32.05	23.32	39.16	34.7	30.14	32.11	41.56	44.31	27.1	1
P32 (10-14-18)	S	S	30.4	21.33	42.18	S	36.82	S	32.94	S	S	0
P33	19.46	27.24	31.02	31.88	35.44	R	31	R	30.7	29.58	27.26	3
P35	41.56	R	R	32.68	33.73	29.86	30.61	26.51	R	36.97	27.78	4
P36 (11/18/18)	36.67	11.67	27.15	R	20.41	27.17	17.91	19.85	20.11	16.63	10.84	5
P37 (11/18/2018)	31.14	9.38	19.01	R	30.37	26.33	28.56	23.37	7.87	27.15	R	5
P38	11.73	29.31	38.15	26.87	33.95	NT	30.34	38.1	33.86	33.9	33.43	1
P39 (*J. ... ...*)	R	25.65	22.58	R	R	35.72	R	45.63	32.35	28.25	R	5
P40	31.38	27.18	28.8	15.59	30.81	R	15.44	R	14.76	30.39	27.12	3
P43.2	34.67	25.46	29.15	24.92	R	R	41.71	27.41	28.35	R	34.56	4
P44	R	ND	33.36	21.93	R	R	39.8	34.37	R	32.48	36.43	5
P46	41.13	27.24	33.1	33.42	33.14	36.25	30.76	41.11	31.82	36.16	30.41	0
P47	37.28	25.52	23.13	16.35	36.95	40.52	37.3	23.49	28.35	37.12	40.24	1
P48.1	R	16.17	21.94	19.56	37.3	47	30.92	27.4	26.61	33.18	15.38	4
P48.2	R	18.71	22.56	24.65	30.32	37.94	38.18	27.1	28.67	29.28	28.17	2
P50.1	31.62	34.42	32.24	25.45	R	46.38	29.8	30.07	28.14	R	25.24	2
P50.2	46.1	28.27	23.7	23.39	23.13	34.17	38.32	25.38	35.03	29.42	19.31	0
P51	R	29.47	24.27	18.12	20.7	R	28.88	20.53	14.55	42.1	24.78	4
P51E	31.16	ND	35.59	31.48	47.1	R	32.95	R	27.64	32.26	25.24	2
P53	R	30.12	35.66	20.96	32.18	R	25.62	24.92	14.69	41.12	31.48	2
P54	44.8	16.5	14.37	21.38	40.23	43.6	27.3	31.93	41.52	37.74	18.39	2
P56C9 A	34.13	38.1	31.65	18.26	26.57	46.9	27.65	24.75	28.91	26.5	21.46	1
P56C9 A	40.17	34.33	33.21	18.32	23.47	36.61	22.15	33.04	22.55	20.67	21.44	1
P58	40.2	S	32.14	25.92	44.16	R	40.55	R	37.31	43.3	35.9	2
P61	13.46	44.66	38.8	R	33.33	R	41.82	35.24	14.82	35.88	35.42	4
P61E	ND	35.38	30.4	31.04	33.22	R	14.16	R	19.71	24.8	22.92	3
P62	S	33.41	23.2	12.35	34.31	S	38.82	R	14.15	36.73	14.22	4
P68A (10/6/1)	33.3	23.82	24.55	R	21.65	34.46	28.95	29.05	22.89	17.96	14.51	3
P71	S	ND	30.12	33.38	33.23	9.26	36.48	31.32	S	36.08	S	2
P71E	S	S	S=Sensitive	S	S	S	S	S	S	S	S	0
ATCC 33560	34.56	28.5	29.55	36.38	34.65	38.45	34.1	27.14	25.6	37.41	28.28	

jejuni because F42 had moldy growth. These results will be confirmed in the future. 15 of the *C. jejuni* were multi drug resistant (MDR), where the bacteria were resistant to 3 or more antibiotics (Table 2).

Conclusions

We noted that differences in AR profiles of the crow isolates from the same date, and site of collection did not always show similar results. It is possible that the crows visited different places during their day time scavenging activities or fed on different substances. Future studies by banding the crows and studying them may shed more light on this.

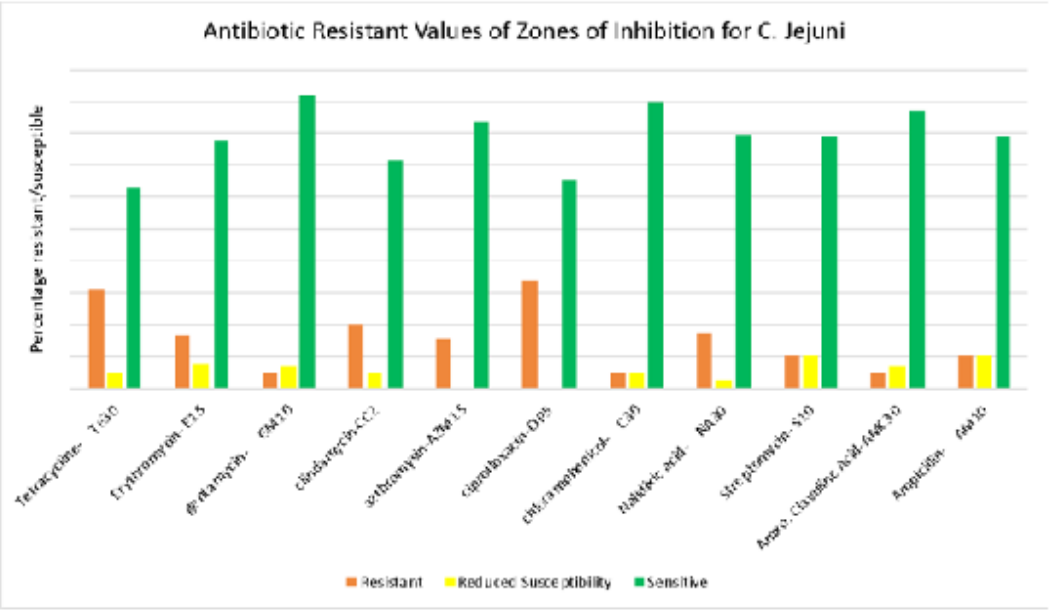
There was also MDR in sixteen out of forty-three total isolates with resistance to least three antibiotics. Other studies have shown that there is high resistance to tetracycline and ciprofloxacin (EFSA, 2014). This correlates with our findings of high resistance to tetracycline and ciprofloxacin.

Lastly, although the *C. jejuni* strains isolated from the crows are not infectious to humans because they lack a functional CDT toxin (Sen et al., 2018, pp. 13), the crows can spread antibiotic resistance through these bacteria, and thus can be of potential threat to human health. Other results from Dr. Sen's laboratory have shown a large number of the bacteria *E. coli* isolated from these fecal samples to also carry antibiotic resistance and several of them were MDR (manuscript submitted).

Acknowledgement

Throughout this course and this paper, the continuous contribution and advice from my peers in this course is evidently shown within this piece. Without their assessment, recommendations, and guidance, the

Figure 3: Percentage of C. jejuni fecal isolates (n = 40) showing susceptibility to 11 selected antimicrobials. The susceptible isolates include only those isolates that were sensitive to the various antibiotics as determined from the CLSI table.



accomplishment of this research and analysis would have been vastly challenging.

I would like to show my gratitude to those who have contributed to this research. My thankfulness for Nidhi Patel for her assistance through the preparation of DNA for some isolates and conducting qPCR to confirm the identity of *C. jejuni*. My appreciation for David Ricci for confirming the measurements of the zones of inhibition and to Dr. Keya Sen, whose knowledge and mentorship have been of great value. Without her continuous support and sincerity throughout the quarter, this research would not have been possible.

References

Altekruse, S. F., Stern, N. J., Fields, P. I., & Swerdlow, D. L. (1999). Campylobacter jejuni—An Emerging Foodborne Pathogen. Emerging Infectious Diseases. *Emerging Infectious Diseases Journal,*5(1), 28-35. https://dx.doi.org/10.3201/eid0501.990104.

Campylobacter. (2018). In World Health Organization. Retrieved from https://www.who.int/news-room/fact-sheets/detail/campylobacter

EFSA and ECDC. (2014). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal.* Retrieved from https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2014.3590

EFSA and ECDC. (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA Journal*. Retrieved from https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5500

Hardy Diagnostics. (1996). Campy CVA Agar. Retrieved from https://catalog.hardydiagnostics.com/cp_prod/content/hugo/campycvaagar.htm

McDermott, F.P., Bodeis, M., S., English, L.L., White, D.G., Walker, R.D., Zhao, S., …,… Wagner, D.D. (2002). Ciprofloxacin Resistance in Campylobacter jejuni Evolves Rapidly in Chickens Treated with Fluoroquinolones. *The Journal of Infectious Diseases,* 185(6), 837-840. https://doi.org/10.1086/339195

Sen, K., Lu, J., Mukherjee, P., Berglund, T., Varughese, E., & Mukhopadhyay, A. K. (2018). Campylobacter jejuni Colonization in the Crow Gut Involves Many Deletions within the Cytolethal Distending Toxin Gene Cluster. *Applied and Environmental Microbiology,* 84(6), 1-13. doi: 10.1128/AEM.01893-17