Journal of Food Safety

Journal of Food Safety ISSN 1745-4565

ISOLATION METHOD (DIRECT PLATING OR ENRICHMENT) DOES NOT AFFECT ANTIMICROBIAL SUSCEPTIBILITY OF *CAMPYLOBACTER* FROM CHICKEN CARCASSES

SCOTT R. LADELY[1], RICHARD J. MEINERSMANN[2,4], JODIE R. PLUMBLEE[2] and PAULA J. FEDORKA-CRAY[3]

[1]U.S. Department of Agriculture, Food Safety Inspection Service, Outbreaks Section of the Eastern Laboratory, Richard B. Russell Agricultural Research Center, 950 College Station Road, Athens, Georgia 30605
[2]U.S. Department of Agriculture, Agriculture Research Service, Bacterial Epidemiology and Antimicrobial Resistance Research Unit, Richard B. Russell Agricultural Research Center, 950 College Station Road, Athens, Georgia 30605
[3]Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, North Carolina 27607

[4]Corresponding author.
TEL: +1-706-546-3236;
FAX: +1-706-546-3616;
EMAIL: rick.meinersmann@ars.usda.gov

Received for Publication October 29, 2015
Accepted for Publication February 24, 2016

doi: 10.1111/jfs.12279

ABSTRACT

To determine if *Campylobacter* isolation method influenced antimicrobial susceptibility results, the minimum inhibitory concentrations (MIC) of nine antimicrobials were compared for 291 pairs of *Campylobacter* isolates recovered from chicken carcass rinse samples using direct plating and an enrichment method. Among the isolates 64.1% were *C. jejuni*, 35.7% were *C. coli*, and 0.2% were *C. lari*. Direct plating yielded significantly less ($P < 0.05$) *C. coli* (21.3%) compared to sample enrichment (50.2%). Antimicrobial resistance was most common for tetracycline (41.4%), nalidixic acid (26.3%), and ciprofloxacin (25.9%). Significantly more ($P < 0.05$) *C. coli* were resistant to nalidixic acid, ciprofloxacin, gentamicin, azithromycin, and erythromycin as compared to *C. jejuni* isolates. Nonparametric bootstrap analysis of antimicrobial MICs showed no significant differences between direct plating and the enrichment method for any of the antimicrobials tested. These data indicate that bacterial isolation method can bias *Campylobacter* species recovery. However, isolation method did not significantly affect antimicrobial susceptibility results of *C. jejuni* or *C. coli* recovered from broiler carcasses.

PRACTICAL APPLICATIONS

To monitor trends in food safety and public health, antimicrobial susceptibility testing of Campylobacter derived from poultry products and infected patients has become common practice in both regulatory food safety and public health programs. Various methods have been employed for Campylobacter recovery including direct plating for enumeration of contamination levels and enrichment protocols for detection of low numbers or injured cells. This study was conducted to determine if the method of Campylobacter isolation from chicken carcass rinsate, direct plating or enrichment, influences antimicrobial susceptibility testing results.

INTRODUCTION

Campylobacter spp. are recognized as important agents of human foodborne gastroenteritis. In 2012, campylobacteriosis was the second most prevalent bacterial foodborne illness reported in the U.S., with an incidence of 14.3 cases per 100,000 population (Gillis *et al.* 2013). Among confirmed cases with species information, the majority were attributed to *C. jejuni* (90%) and *C. coli* (8%). Poultry is a primary reservoir of *Campylobacter*. Surveys conducted by the U.S. Department of Agriculture (USDA), Food Safety Inspection

Service (FSIS), found 46.6% of young chickens (Anonymous 2010) and 21.4% of chicken parts contaminated with *Campylobacter* spp. (Anonymous 2014).

Mishandling raw poultry, cross-contamination of ready-to-eat foods, and consumption of undercooked poultry are important risk factors for human *Campylobacter* infection (Engberg 2006; Luber 2009). Most cases of gastroenteritis result in a self-limiting diarrheal disease that does not require antimicrobial therapy. However, prolonged duration of illness, or altered immune function in some individuals, may warrant antimicrobial therapy (Aarestrup and Engberg 2001; Allos 2001). Macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) are usually used in the treatment of human *Campylobacter* infections. To monitor trends in public health and food safety, antimicrobial susceptibility testing of pathogens derived from infected patients and food products has become common practice in both public health and regulatory food safety programs.

Previous studies have shown that choice of plating medium (Carrillo *et al.* 2005; Oyarzabal *et al.* 2005) and enrichment (Carrillo *et al.* 2005) may influence prevalence and diversity of *Campylobacter* recovery from poultry samples. However, information regarding the possible effects of isolation method on antimicrobial susceptibility is lacking. The objective of this study was to determine if the method of *Campylobacter* isolation from chicken carcass rinsate, direct plating or enrichment, influences antimicrobial susceptibility testing results.

MATERIALS AND METHODS

Campylobacter Isolation Methods

Sample paired *Campylobacter* isolates ($n = 582$) were recovered using two isolation methods: a direct plating method for enumeration ($n = 291$) and an enrichment method ($n = 291$) used for increased sensitivity and recovery of potentially injured cells. *Campylobacter* isolates were recovered from chicken carcass rinse samples collected between July 2011 and June 2012. Briefly, for quantitative detection and enumeration, 1 mL of chicken carcass rinsate was spread-plated onto four Campy-Cefex (Stern et al. 1992) agar plates (250 µL/plate) and 0.1 mL was spread onto two additional (duplicates) plates. Plates were incubated microaerobically (5% O_2, 10% CO_2 and 85% N_2) for 48 h at 42C. For enrichment, 30 mL of the carcass rinsate was added to an equal volume of double-strength blood-free *Campylobacter* enrichment broth (Bolton formulation) (Acumedia, Neogen Corp., Lansing, MI) and incubated microaerobically for 48 h at 42C. Broth was then streaked onto Campy-Cefex agar and plates were incubated as described above. Typical colonies were confirmed by observation of cellular morphology and motility using phase-contrast microscopy and by latex agglutination (PanBio-Campy, Scimedx Corp., Denville, NJ). *Campylobacter* species was determined using the *Campylobacter* BAX® PCR (DuPont Nutrition and Health; Wilmington, DE) according to manufacturer's instructions.

Antimicrobial Susceptibility Testing

Sample paired *Campylobacter* isolates ($n = 582$), recovered from 291chicken carcass rinse samples by direct plating and an enrichment method, were evaluated for susceptibility to a panel of nine antimicrobials used by the National Antimicrobial Resistance Monitoring System (NARMS) program (http://www.ars.usda.gov/Main/docs.htm?docid=6750). Minimal inhibitory concentrations (MIC) were determined by broth microdilution using the Sensititre system (ThermoFisherScientific, Waltham, MA) and recommended quality-control organisms. MICs were interpreted according to Clinical and Laboratory Standards Institute (CLSI 2010) guidelines when available. Otherwise, interpretive criteria as established by the NARMS were used. Antimicrobial breakpoints (MICs) were as follows: azithromycin ≥ 8 µg mL^{-1}; ciprofloxacin ≥ 4 µg mL^{-1}; clindamycin ≥ 8 µg mL^{-1}; erythromycin ≥ 32 µg mL^{-1}; florfenicol > 4 µg mL^{-1}; gentamicin ≥ 8 µg mL^{-1}; nalidixic acid ≥ 64 µg mL^{-1}; telithromycin ≥ 16 µg mL^{-1}; and tetracycline ≥ 16 µg mL^{-1}.

Statistical Analysis

Nonparametric bootstrap analysis (SAS, ver. 9.3, SAS Institute Inc., Cary, NC) was used to determine if there were significant differences in antimicrobial MICs between *Campylobacter* isolation methods and Fisher's exact probability test was used to determine if there were significant differences ($P < 0.05$) in antimicrobial susceptibility between *Campylobacter* species.

RESULTS AND DISCUSSION

The intent of this study was to determine if *Campylobacter* isolation method influenced antimicrobial susceptibility testing results. Sample paired isolates ($n = 582$), recovered from 291 chicken carcass rinse samples by direct plating and an enrichment method, were identified to species and evaluated for antimicrobial susceptibility. Overall, the recovery of *Campylobacter* spp. was 64.1% *C. jejuni* ($n = 373$), 35.7% *C. coli* ($n = 208$), and 0.2% *C. lari* ($n = 1$). The same species of *Campylobacter* was recovered by both direct plating and enrichment for 65.6% ($n = 191$) of the samples, while 34.4% of the samples ($n = 100$) yielded two different species. Others have recovered multiple *Campylobacter* species from poultry samples (Kramer *et al.* 2000; Carrillo *et al.* 2014; Oyarzabal *et al.* 2005), but at much lower frequencies (2.4% −12.1% of samples).

TABLE 1. NUMBER (%) OF *CAMPYLOBACTER* ISOLATES RESISTANT TO ANTIMICROBIALS BY SPECIES AND ISOLATION METHOD*†

	C. jejuni			*C. coli*		
	Direct plating	Enrichment	Total	Direct plating	Enrichment	Total
Antimicrobial	($n = 229$)	($n = 144$)	($n = 373$)	($n = 62$)	($n = 146$)	($n = 208$)
Tetracycline	99 (43.2)	67 (46.5)	166 (44.5)	24 (38.7)	51 (34.9)	75 (36.1)
Nalidixic acid	54 (23.6)^{AB}	24 (16.7)^{A}	78 (20.9)^{A}	20 (32.3)^{BC}	54 (37.0)^{C}	74 (35.6)^{C}
Ciprofloxacin	53 (23.1)^{AB}	23 (16.0)^{A}	76 (20.4)^{A}	20 (32.3)^{BC}	54 (37.0)^{C}	74 (35.6)^{C}
Gentamicin	1 (0.4)^{A}	0 (0)^{A}	1 (0.3)^{A}	3 (4.8)^{B}	4 (2.7)^{AB}	7 (3.4)^{B}
Azithromycin	0 (0)	0 (0)	0 (0)^{A}	2 (3.2)	5 (3.4)	7 (3.4)^{B}
Erythromycin	0 (0)	0 (0)	0 (0)^{A}	2 (3.2)	5 (3.4)	7 (3.4)^{B}
Clindamycin	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.5)
Telithromycin	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	1 (0.5)
Florfenicol	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

*Antimicrobial breakpoints (MICs): azithromycin $\geq$8 μg mL^{-1}; ciprofloxacin $\geq$4 μg mL^{-1}; clindamycin $\geq$8 μg mL^{-1}; erythromycin $\geq$32 μg mL^{-1}; florfenicol >4 μg mL^{-1}; gentamicin $\geq$8 μg mL^{-1}; nalidixic acid $\geq$64 μg mL^{-1}; telithromycin $\geq$16 μg mL^{-1}; and tetracycline $\geq$16 μg mL^{-1}.
†One *C. lari* isolate (resistant to ciprofloxacin and nalidixic acid) was not included in Table 1.
^{ABC}Values within rows without common superscripts are significantly different ($P < 0.05$) by Fisher's exact test.

Campylobacter isolation method affected the species of *Campylobacter* recovered. Direct plating carcass rinsate onto Campy-Cefex agar yielded significantly less ($P < 0.05$) *C. coli* compared to enrichment of the rinsate followed by plating on Campy-Cefex. Direct plating resulted in the recovery of 21.3% *C. coli* ($n = 62$) and 78.7% *C. jejuni* ($n = 229$) compared to 50.2% *C. coli* ($n = 146$) and 49.5% *C. jejuni* ($n = 144$) recovered using the enrichment method. For 31.6% ($n = 92$) of the samples, *C. jejuni* was recovered by direct plating and *C. coli* was recovered by the enrichment method; conversely, *C. coli* was recovered by direct plating and *C. jejuni* by enrichment in only 2.4% ($n = 7$) of the samples. In one sample, *C. coli* was isolated by direct plating and *C. lari* was recovered by enrichment. Other researchers (Kramer *et al.* 2000; Carrillo *et al.* 2014) using similar plating and enrichment media have reported that in cases of multispecies contamination, *C. coli* also was more likely to be recovered after enrichment than by direct plating. Among samples with multispecies contamination, Carrillo *et al.*, (2014) recovered more *C. coli* after 48 h enrichment compared to 24 h, indicating that *C. coli* may out compete *C. jejuni* after 48 h of enrichment. They (Carrillo *et al.* 2014) suggested use of shorter enrichment times and selecting multiple colonies from multiple types of selective agar plates to increase the likelihood of identifying all *Campylobacter* spp. associated with a food product. Others, however, have noted that attempts to reduce enrichment time from 48 to 24 h decrease the recovery of *Campylobacter* spp. from poultry meat samples (Liu *et al.* 2009).

Sample paired *Campylobacter* isolates ($n = 582$) recovered from 291 chicken carcass rinse samples by direct plating and an enrichment method were evaluated for susceptibility to a panel of nine antimicrobials. Prevalence of antimicrobial resistance by species and isolation method are shown in Table 1. Resistance to tetracycline was most common and was similar for *C. jejuni* and *C. coli* isolates (44.5% and 36.1%, respectively). In contrast, significantly more ($P < 0.05$) *C. coli* compared to *C. jejuni* were resistant to quinolones (35.6% and 20.9% resistance to nalidixic acid, and 35.6% and 20.4% resistance to ciprofloxacin for *C. coli* and *C. jejuni*, respectively), macrolides (3.4% and 0 resistance to azithromycin and erythromycin for *C. coli* and *C. jejuni*, respectively) and gentamicin (3.4% and 0.3% for *C. coli* and *C. jejuni*, respectively). No isolates were resistant to florfenicol. These results are not uncommon as *C. coli* tend to be more resistant than *C. jejuni*, regardless of the host species (Bywater *et al.* 2004; Englen *et al.* 2007; Torralbo *et al.* 2015).

Antimicrobial resistance profiles of *Campylobacter* isolates by species are provided in Table 2. Two-hundred-sixty isolates (44.7%) were susceptible to all antimicrobials tested. Significantly more ($P < 0.05$) *C. jejuni* isolates were susceptible to all antimicrobials tested (48.3%) compared to *C. coli* isolates (38.5%). The most prevalent resistance profile was to a single antimicrobial, tetracycline. Significantly more ($P < 0.05$) *C. jejuni* isolates were resistant to tetracycline only (30.6%) as compared to *C. coli* isolates (20.7%). Resistance to quinolones (nalidixic acid and ciprofloxacin) was the next most frequently observed resistance profile with significantly more *C. coli* (24.5%) than *C. jejuni* (7.2%) isolates resistant to these two antimicrobials. Few isolates were resistant to two or more classes of antimicrobials. Resistance to quinolones and tetracycline accounted for the majority of multi-drug resistance (13.1% and 9.6% of *C. jejuni* and *C. coli* isolates, respectively), while only four *C. coli* isolates (0.7% of isolates examined) were resistant to three classes of antimicrobials.

Table 3 shows antimicrobial resistance prevalence by isolation method. For each antimicrobial tested, nonparametric bootstrap analysis of sample paired isolates showed no significant differences between the MIC means of direct plating

TABLE 2. ANTIMICROBIAL RESISTANCE PATTERNS OF *CAMPYLOBACTER* ISOLATES BY SPECIES

No. Antimicrobials	Resistance pattern†	No. (%) of isolates with resistance pattern*	
		C. jejuni ($n = 373$)	*C. coli* ($n = 208$)
0	Pan-susceptible	180 (48.3)^{A}	80 (38.5)^{B}
1	TC	114 (30.6)^{A}	43 (20.7)^{B}
2	AZ EM	0 (0)	1 (0.5)
2	NA TC	2 (0.5)	0 (0)
2	CI NA	27 (7.2)^{A}	51 (24.5)^{B}
2	GM TC	1 (0.3)^{A}	6 (2.9)^{B}
3	AZ EM TC	0 (0)	2 (1.0)
3	CI NA TC	49 (13.1)	20 (9.6)
4	AZ CI EM NA	0 (0)	1 (0.5)
4	AZ CM EM TC	0 (0)	1 (0.5)
4	AZ EM TL TC	0 (0)	1 (0.5)
4	CI GM NA TC	0 (0)	1 (0.5)
5	AZ CI EM NA TC	0 (0)	1 (0.5)

*One *C. lari* isolate resistant to CI and NA was not included in Table 2.

†AZ, azithromycin; CI, ciprofloxacin; CM, clindamycin; EM, erythromycin; GM, gentamicin; NA, nalidixic acid; TC, tetracycline; TL, telithromycin.

^{AB}Values within rows without common superscripts are significantly different ($P < 0.05$) by Fisher's exact test.

or enrichment. These results indicate that comparing antimicrobial susceptibility results between the two *Campylobacter* isolation methods is unbiased. However, significantly more *C. coli* were recovered by sample enrichment compared to direct plating of carcass rinsate. The increased prevalence of *C. coli* associated with enrichment observed here was likely the result of growth phase differences between species. In addition, the *C. coli* isolates recovered were significantly more resistant than *C. jejuni* isolates. Hypothetically, in geographic locations where *C. coli* is more prevalent in poultry, *Campylobacter* isolation method, particularly 48h enrichment, could result in increased detection of resistant strains due to enrichment bias for *C. coli*. Regardless, no single isolation method is optimal for *Campylobacter* detection in all foods and selectivity differences among enrichment and plating media have been observed (Ng *et al*. 1985; Oyarzabal *et al*. 2005; Carrillo *et al*. 2014). In this study, the formulation of plating and enrichment media shared selective components (cefoperazone and cycloheximide) and a common plating media was used for both isolation methods. These data indicate that bacterial isolation method can bias *Campylobacter* species recovery. However, isolation method did not significantly affect antimicrobial susceptibility results of *C. jejuni* or *C. coli* recovered from broiler carcasses.

TABLE 3. NUMBER (%) OF *CAMPYLOBACTER* ISOLATES RESISTANT TO ANTIMICROBIALS BY ISOLATION METHOD*†

Antimicrobial	Direct plating ($n = 291$)	Enrichment ($n = 291$)	Total ($n = 582$)
Tetracycline	118 (40.5)	123 (42.3)	241 (41.4)
Nalidixic acid	74 (25.4)	79 (27.1)	153 (26.3)
Ciprofloxacin	73 (25.1)	78 (26.8)	151 (25.9)
Gentamicin	4 (1.4)	4 (1.4)	8 (1.4)
Azithromycin	2 (0.7)	5 (1.7)	7 (1.2)
Erythromycin	2 (0.7)	5 (1.7)	7 (1.2)
Clindamycin	0 (0)	1 (0.3)	1 (0.2)
Telithromycin	0 (0)	1 (0.3)	1 (0.2)
Florfenicol	0 (0)	0 (0)	0 (0)

*Nonparametric bootstrap analysis of antimicrobial MICs showed no significant differences between direct plating and the enrichment method for any of the antimicrobials tested.

†Antimicrobial breakpoints (MICs): azithromycin $\geq$8 μg mL^{-1}; ciprofloxacin $\geq$4 μg mL^{-1}; clindamycin $\geq$8 μg mL^{-1}; erythromycin $\geq$32 μg mL^{-1}; florfenicol >4 μg mL^{-1}; gentamicin $\geq$8 μg mL^{-1}; nalidixic acid $\geq$64 μg mL^{-1}; telithromycin $\geq$16 μg mL^{-1}; and tetracycline $\geq$16 μg mL^{-1}.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Zaid Abdo, U.S. Department of Agriculture, Agriculture Research Service, and Sara Byers (student) in the Department of Statistics at the University of Georgia for assisting in bootstrap analysis.

REFERENCES

AARESTRUP, F.M. and ENGBERG, J. 2001. Antimicrobial resistance of thermophilic *Campylobacter*. Vet. Res. *32*, 311–321.

ALLOS, B.M. 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. Clin. Infect. Dis. *32*, 1201–1206.

ANONYMOUS. 2010. The Nationwide Microbiological Baseline Data Collection Program: Young Chicken Survey July 2007–June 2008. http://www.fsis.usda.gov/wps/wcm/connect/deab6607-f081-41a4-90bf-8928d7167a71/Baseline_Data_Young_Chicken_2007-2008.pdf?MOD=AJPERES (accessed 22 April 2015).

ANONYMOUS. 2014. The Nationwide Microbiological Baseline Data Collection Program: Raw Chicken Parts Survey January 2012–August 201.2 http://www.fsis.usda.gov/wps/wcm/connect/a9837fc8-0109-4041-bd0c-729924a79201/Baseline_Data_Raw_Chicken_Parts.pdf?MOD=AJPERES (accessed 22 April 2015).

BYWATER, R., DELUYKER, H., DEROOVER, E., DE JONG, A. MARION, H., MCCONVILLE, M., ROWAN, T., SHRYOCK, T, SHUSTER, D., THOMAS, V., VALLE, M. and WALTERS D.J. 2004. A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. J. Ant. Chem. *54*, 744–754.

CARRILLO, C.D., PLANTE, D., IUGOVAZ, I., KENWELL,R., BELANGER, G., BOUCHER, F., POULIN, N. and TROTTIER, Y. 2014. Method-dependent variability in determination of prevalence of *Campylobacter jejuni* and *Campylobacter coli* in Canadian retail poultry. J. Food Prot. *77*, 1682–1688.

CLSI. 2010. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline, 2nd ed. *CLSI document M45-A2*. CLSI, Wayne, PA.

ENGBERG, J. 2006. Contributions to the epidemiology of *Campylobacter* infections. A review of clinical and microbiological studies. Dan. Med. Bull. *53*, 361–389.

ENGLEN, M.D., HILL, A.E., DARGATZ, D.A., LADELY, S.R., and FEDORKA-CRAY, P.J. 2007. Prevalence and antimicrobial resistance of *Campylobacter* in US dairy cattle. J. Appl. Microbiol. *102*, 1570–1577.

GILLIS, D., A. B. CRONQUIST, and M. CARTTER. 2013. Incidence and trends of infection with pathogens transmitted commonly through food–foodborne diseases active surveillance network, 10 U.S. sites, 1996-2012, MMWR. *62*, 283–287.

KRAMER, J.M., FROST, J.A., BOLTON, F.J., and WAREING, D.R. 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. J. Food Prot. *63*, 1654–1659.

LIU, L., HUSSAIN, S.K., MILLER, R.S., and OYARZABAL, O.A. 2009. Efficacy of Mini Vidas for the detection of *Campylobacter* spp. from retail broiler meat enriched in Bolton broth, with or without the supplement of blood. J. Food Prot. *72*, 2428–2432.

LUBER, P. 2009. Cross-contamination versus undercooking of poultry meat or eggs-which risks needs to be managed first? Int. J. Food Microbiol. *134*, 21–28.

NG, L., STILES, M.E. and TAYLOR, D.E. 1985. Inhibition of *Campylobacter coli* and *Campylobacter jejuni* by antibiotics used in selective growth media. J. Clin. Microbiol. *22*, 510–514.

OYARZABAL, O.A., MACKLIN, K.S., BARBAREE, J.M. and MILLER, R.S. 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Appl. Environ. Microbiol. *71*, 3351–3354.

STERN, N.J., WOJTON, B. and KWIATEK, K. 1992. A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. J. Food Prot. *55*, 514–517.

TORRALBO, A., BORGE, C., GARCIA-BOCANEGRA, I., MERIC, G., PEREA, A. and CARBONERO, A. 2015. Higher resistance of *Campylobacter coli* compared to *Campylobacter jejuni* at chicken slaughterhouse. Comp. Immunol. Microbiol. Infect. Dis. *39*, 47–52.