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Research Article

Prevalence of Antimicrobial Resistance (AMR) in Bacteria Isolated from Farm Animals, Wildlife, and Food Samples in the Eastern United States between 2007 and 2013

Chyer Kim[1]*, Antoinette Torres[2], Wesley Smith[3], Ariana Kulinczenko[3], Steven Pao[4], Stephen Wildeus[1], Matthew Ettinger[5], Karen Gruszynski[6] and Crystal Wynn[3]

[1]Agricultural Research Station, Virginia State University, Petersburg, USA

[2]Department of Biology, Virginia State University, Petersburg, USA

[3]Department of Family and Consumer Sciences, Virginia State University, Petersburg, USA

[4]Department of Food Science and Nutrition, California State University, Fresno, USA

[5]Office of Radiological Health, Virginia Department of Health, Richmond, Virginia, USA

[6]Office of Epidemiology, Virginia Department of Health, Richmond, Virginia, USA

***Corresponding Author**: Chyer Kim, Agricultural Research Station, Virginia State University, Petersburg, USA.

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Abstract

Due to the extensive use of antimicrobial agents in human and agricultural practices, antimicrobial-resistant (AMR) bacteria have become a critical worldwide health issue. This study evaluated the prevalence of antimicrobial resistance in *E. coli, Salmonella* and *Campylobacter* isolates obtained from animal and food samples between 2007 and 2013. A total of 265 bacterial isolates consisting of 66 *E. coli*, 121 *Salmonella* and 78 *Campylobacter* were tested with the Kirby-Bauer disc diffusion method for their susceptibility to 12 antimicrobials representing nine different categories. Tetracycline (TCY) was the antimicrobial agent that showed the highest frequency of resistance among *E. coli* and *Salmonella* isolates (62.1% and 13.2%, respectively), while the most frequent trimethoprim-sulfamethoxazole (SXT) resistance was detected among *Campylobacter* isolates (58.3%). Approximately 19.7% of *E. coli,* 5.0% of *Salmonella* and 23.1% of *Campylobacter* isolates exhibited resistance to three or more categories of antimicrobials, meeting criteria for multi-drug resistance (MDR). Eighty-three percent of *E. coli*, 31% of *Salmonella* and 94% of *Campylobacter* isolates were found to be resistant to at least one of the antimicrobials tested in this study. While one strain of *Salmonella* demonstrated resistance to eight antimicrobials, it was still susceptible to meropenem (MEM), amikacin (AMK), ciprofloxacin (CIP), and nalidixic acid (NAL). In addition, two *Campylobacter* isolates demonstrated intermediate resistance to 10 antimicrobials. Findings in this study clearly demonstrate different patterns of resistance among bacterial species that present in our environment and further emphasize the need for judicious and careful use of antimicrobials in human and agricultural practices to help reduce future manifestations of MDR bacteria in food-borne illnesses.

Keywords: *E. coli; Salmonella; Campylobacter; AMR; Prevalence*

Introduction

The use of antimicrobials in human and agricultural practices has led to large-scale dissemination of antimicrobial resistant (AMR) bacteria, in the environment posing a serious health risk worldwide [3,4,15,24,38]. The US Centers for Disease Control and Prevention (CDC) estimates that more than 2 million people are infected with AMR bacteria each year, and at least 23,000 people die as a direct result of these infections [11]. In light of the World Health Organization's call for improved surveillance of AMR [51] and the FDA's continuous effort to enhance and strengthen antimicrobial surveillance through the National Antimicrobial Resistance Monitoring System (NARMS), multi-drug resistance (MDR) bacteria are gaining more and more attention worldwide [5].

With the continued reports of food-borne illnesses in the US, there is a strong demand for further research on bacterial strains isolated from contaminated food and their potential resistance to antimicrobials. More often than not food-borne illnesses are over-treated with the use of antimicrobials resulting in a spread of MDR bacterial strains infecting both humans and animals [32]. Several studies have linked this spread of resistance with the use of antimicrobials in veterinary medicine as feed additives and the transfer of resistance isolates through the foods of animal origin to humans [19,30,32,39,41]. The government is demanding a reduction in the usage of antimicrobials in veterinary and human medicine practices [52] as a result. Brown., *et al.* [10] indicated that the primary source of antimicrobials in the general environment is the excretion of partially metabolized antimicrobials by humans and animals.

Increased international attention to the risks associated with antimicrobial use in animal production has helped spur the development of numerous surveillance systems and networks [49]. The US government requires the monitoring of trends in AMR among foodborne bacteria collected from humans, retail meats and food-producing animals [22]. In addition, President Obama recently declared federal policy for 'responsible use' of antimicrobials in food production [52]. MDR bacteria are an increasing health concern worldwide and there is worry of widespread prevalence of AMR bacteria in our environment. As a consequence, NARMS monitors AMR of food-borne pathogens and identifies the sources and magnitude of AMR in the food supply. Because *Salmonella* and *Campylobacter* are the leading bacterial causes of foodborne illness, NARMS particularly monitors these bacteria to determine their resistance to various antimicrobials used in human and veterinary medicine. *E. coli* is also included, primarily to help track the occurrences and spread of resistance in the environment and in food products [22]. Furthermore, the level of AMR in *E. coli* is considered a good indicator of the selection pressure exerted by antimicrobial use [1,46]. Due to concerns about environmental and foodborne AMR, this study aims to evaluate the prevalence of antimicrobial resistance of *E. coli, Salmonella* and *Campylobacter* isolates obtained from small ruminants, wildlife and food samples in the Eastern United States between 2007 and 2013.

Materials and Methods

Bacterial isolates used: A total of 265 isolates were used in this study consisting of 66 *E. coli* isolates, 121 *Salmonella* isolates, and 78 *Campylobacter* isolates. These isolates were obtained from prior research studies conducted during a 7-year period (2007 – 2013). The *E. coli* isolates were obtained from meat purchased from Internet-based and local retail markets (referred to as 'food study', 31). Of the *Salmonella* isolates, 89 were obtained from fecal samples of wildlife ('wildlife study', 26-27) and 32 were obtained from fecal samples of farm-reared small ruminants and wild-living birds ('farm study', 37). The *Campylobacter* isolates also were obtained from the farm study. *Salmonella* and *E. coli* isolates maintained in tryptic soy broth (TSB) containing 20% glycerol at -80°C were revived using Mueller-Hinton agar (MHA, Becton, Dickinson and Company, Sparks, MD), while MHA supplemented with 5% sheep blood (MHAB, Becton, Dickinson and Company) was used to revive *C. jejuni* isolates.

Microbiological isolation and identification: Bacterial isolation from the samples and their identification were previously done following AOAC-approved or performance tested methods [42-44]. In brief, for *E. coli*, a loopful of culture from lauryl sulfate tryptose broth that produced gas was transferred to EC broth containing 4-methylumbelliferyl-b-D-glucuronide (EC-mug). After incubation for 24 to 48 h at 45.5°C, a loopful of culture from EC-mug tubes with growth and fluorescence under long-wave UV light at 365 nm was streaked on eosine–methylene blue agar; purple colonies (with or without a green metallic sheen) were identified by API 20 E® test strip for *E. coli.* For *Salmonella*, each sample was pre-enriched in buffered peptone water at 36°C for 20h, followed by enrichment in Rappaport–Vassiliadis (RV) broth at 42°C for 18h and post-enrichment in mannose (M) broth at 36°C for 7 h before the *Salmonella* enzyme-linked immunosorbent assay (ELISA; Tecra, Frenchs Forest, Australia) was performed [36]. One loop of ELISA-positive samples of RV and/or M broth was streaked on xylose–lysine–deoxycholate agar (Becton, Dickinson and Company, Sparks, MD, USA) for isolation. Typical colonies (red colonies with or without black centers) were isolated, and at least one isolate was identified to genus level using Gram staining and API 20 E® test strip (bioMérieux, Marcy l'Etoile, France). For *Campylobacter,* each sample was enriched in Bolton's broth with cefoperazone, vancomycin, trimethoprim, and cycloheximide (Antibiotic Supplements SR0183, Oxoid Ltd., Basingstoke, Hampshire, England) microaerobically using AnaeroPack System with Pack-MicroAero (Mitsubishi Gas Chemical, New York, NY) at 42°C for 44 - 48h. A loopful of the

enrichment was then streaked onto a modified *Campylobacter* blood-free selective agar with cefoperazone and amphotericin B (Antibiotic Supplements SR0155, Oxoid Ltd.) for an additional 44-48h incubation at 42°C. Isolates showing a *Campylobacter*-like morphology on blood agar plates, Gram-negative seagull-like cell morphology under light microscopy, and positive reactions in catalase and oxidase tests were considered *Campylobacter* spp. [8]. Isolates subsequently testing positive for hippurate-hydrolysis (Hippurate disk, Remel, Lenexa, KS) were identified as *C. jejuni* [9,40].

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing of *E. coli* and *Salmonella* isolates was performed on MHA by the Kirby-Bauer disk diffusion method [28] following the Clinical and Laboratory Standards Institute (CLSI) guidelines [14] while MHAB was used for *C. jejuni* isolates. The isolates were tested for their susceptibility against 12 antimicrobials representing 9 different antimicrobial categories (Table 1). The MHA plates inoculated with *E. coli* and *Salmonella* were placed with antimicrobial-impregnated disks and incubated at 37°C for 24h while the MHAB plates inoculated with *Campylobacter* were incubated at 37°C under micro-aerobic conditions for 24h. Antimicrobial susceptibility (such as "resistant," "intermediate," and "susceptible") was interpreted in accordance with interpretive criteria provided by the National Committee of Clinical Laboratory Standards (NCCLS) recommendations [14]. In addition, bacteria that were either resistant or intermediate to antimicrobials were considered to be non-susceptible and the ones resistant to at least one microbial agent in three or more antimicrobial categories were defined as MDR [22,33]. *E. coli* ATCC 25922 was used as a control strain for the performance of antimicrobials used in this study.

Antimicrobial category	Antimicrobial agent and its abbreviation	Concentration (µg/disk)	Zone diameter (mm)/ Interpretive criteria*		
			S	I	R
Penicillins	Ampicillin (AMP)	10	≥ 17	14 - 16	≤ 13
β - lactamase inhibitor combinations	Amoxicillin - clavulanic acid (AMC)	30	≥ 18	14 - 17	≤ 13
Carbapenems	Meropenem (MEM)	10	≥ 23	20 - 22	≤ 19
Aminoglycosides	Amikacin (AMK)	30	≥ 17	15 - 16	≤ 14
	Gentamicin (GEN)	10	≥ 15	13 - 14	≤ 12
	Streptomycin (STR)	10	≥ 15	12 - 14	≤ 11
	Tobramycin (TOB)	10	≥ 15	13 - 14	≤ 12
Tetracyclines	Tetracycline (TCY)	30	≥ 15	12 - 14	≤ 11
Fluoroquinolones	Ciprofloxacin (CIP)	5	≥ 21	16 - 20	≤ 15
Quinolones	Nalidixic acid (NAL)	30	≥ 19	14 - 18	≤ 13
Phenicols	Chloramphenicol (CHL)	30	≥ 18	13 - 17	≤ 12
Folate pathway inhibitors	Trimethoprim - sulfamethoxazole (SXT)	25	≥ 16	11 - 15	≤ 10

Table 1: *Summary of antimicrobial categories, agents, concentrations, and interpretive criteria used in this study [14].*

**S: susceptible, I: intermediate, and R: resistant to antimicrobial agent*

Results and Discussion

The number of *E. coli* isolates from the food study with resistance to the 12 antimicrobials tested in this study are summarized in Table 2. Among the 66 isolates, 55 (83.3%) were resistant to one or more antimicrobials. Only one (1.5%) isolate was susceptible to all tested antimicrobials, indicating 98.5% of *E. coli* isolates were non-susceptible (either resistant or intermediate) to one or more antimicrobials (data not shown). The present survey revealed that five (7.6%) isolates were non-susceptible to more than eight antimicrobials. Those isolates were from either Internet- or locally-acquired lamb meat with varying API profiles (5044572, 5144562, and 5144572) and no isolates had matching pulsed field gel electrophoresis (PFGE) profiles. Multi-drug resistance (MDR) was found in 13 (19.7%) isolates of which 69.2% were lamb and 30.8% were goat. Only two (15.4%) of those isolates came from locally acquired meats.

Type of bacterial isolates[b]	Quantity of antimicrobial agents to which bacterial isolates demonstrate resistance[c]									MDR[d]
	1	2	3	4	5	6	7	8	9	
E. coli	20	21	11	2	1	0	0	0	0	13
Salmonella	26	4	3	1	2	0	0	1	0	6
from wildlife	19	2	2	1	2	0	0	1	0	6
from farm	7	2	1	0	0	0	0	0	0	0
Campylobacter	28	24	6	5	6	0	2	1	1	18

Table 2: *Number of bacterial isolates obtained from animal and food samples between 2007 and 2013 that exhibit resistance to one or more antimicrobial agents[a].*

[a] *Susceptibility categorization was carried out in accordance with interpretive criteria provided by the National Committee of Clinical Laboratory Standards (NCCLS) recommendations [14].*

[b] *Total number of isolates tested were 66 for E. coli, 121 for Salmonella (with 89 from the wildlife study and 32 from the farm study), and 78 for Campylobacter.*

[c] *These values designate the quantity of antimicrobials to which each type of bacterial isolates showed resistance; the specific antimicrobial(s) to which any single isolate is resistant may differ.*

[d] *Quantity of multi-drug resistant isolates. See the text for the definition of MDR.*

The susceptible, intermediate, and resistant patterns of *E. coli* isolates in relation to the antimicrobials tested in this study are presented in Figure 1. Resistance to TCY was the most common in 41 (62.1%) isolates, followed by AMP (50%), TOB (16.7%), and STR (10.6%). No resistance was found to MEM, AMK, CIP, CHL, and SXT, yet each showed intermediate resistance of 3.0, 19.7, 0.0, 4.5, and 1.5%, respectively. In other words, all *E. coli* isolates were susceptible to CIP only. Similar to our results, high AMR rates in environmental *E. coli* isolates have been reported by Du Plessis., *et al*. [18] for TCY (33.3 – 60.0%) and AMP (25.0 – 60.0%). Another study [38] conducted in Austria also found high resistance rates to TCY (57%) and AMP (18%) in *E. coli* isolated from sewage and sludge. Moreover, Donado-Godoy., *et al*. [16] reported similar results with very high resistance to TCY (92.5%) and AMP (40.0%) in *E. coli* isolates from Colombian poultry meat. These authors speculated that the highest prevalence of resistance to TCY could be associated with the use of chlortetracycline in feed as a growth promoter, which is allowed on broiler farms in Colombia. *E. coli* isolates obtained from clinical specimens collected from hospitals in Sudan [29] also showed high prevalence of resistance to TCY (77.1%), suggesting the global prevalence of AMR *E. coli*. While this is addressed by Doyle., *et al*. [17], the variability among countries and regions in prevalence and diversity of resistance to different antimicrobials may be due to substantive differences in antimicrobial usage and practices. The overall lack of resistance to MEM found in the current study, when combined with low resistance to AMK reported by Donado-Godoy., *et al*. [16], suggest that these antimicrobials may be still effective for treatment of *E. coli*, which is an opportunistic human pathogen [22].

Among the 121 *Salmonella* isolates, 37 (30.6%) isolates [27 (30.3%) isolates from the wildlife study and 10 (31.3%) isolates from the farm study] were resistant to one or more antimicrobials, indicating a similar prevalence of AMR in *Salmonella* isolates obtained from both wildlife and farm animals (Table 2). Our study demonstrated that only nine (7.4%) isolates were susceptible to all tested antimicrobials, indicating that 92.6% of *Salmonella* isolates were non-susceptible (data not shown). Findings from the farm study again revealed a higher prevalence of *Salmonella* in sheep (25 Isolates, 80.6%) than in goats (6 Isolates, 19.4%), which is similar to our prior observations of *E. coli.* Although a direct correlation is speculative, it is noteworthy that the foraging behavior of sheep and goats differ in ways that may help to explain the observed differences in AMR in our isolates. Goats are browsers and prefer to nibble the tops of plants while sheep are grazers and prefer to eat plants down to the soil surface [7,13,37]. Interestingly, the isolate showing the highest level of AMR, confirmed

as *Salmonella* senftenberg, was obtained from a gull and displayed resistance to 8 antimicrobials. This finding corroborates other research [26,27] suggesting that birds could be potential vectors for dispersal of AMR and pathogenic microbes.

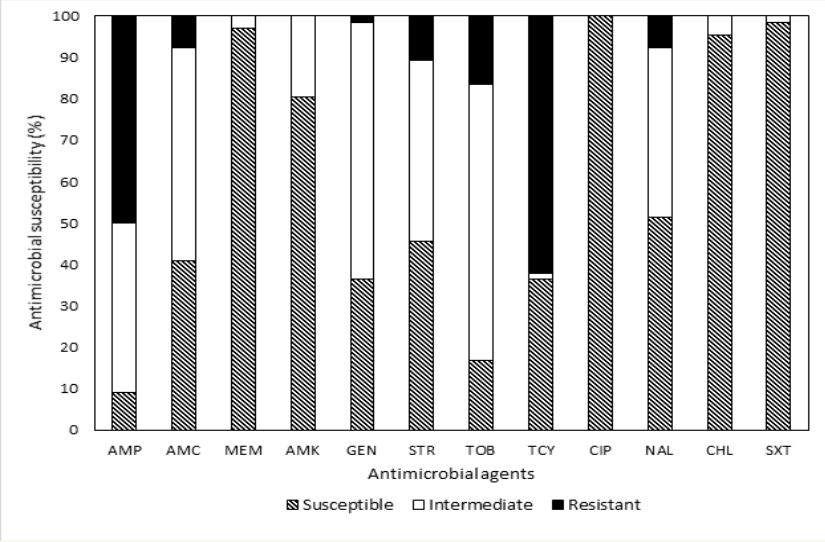

Figure 1: *Prevalence of resistance to 12 antimicrobial agents in 66 E. coli isolates.*

MDR was found in six (6.7%) isolates obtained from the wildlife study, indicating a higher prevalence of MDR rates in *Salmonella* from wildlife than in (0.0%) farm-raised small ruminant animals. Although one isolate from a wild bird (European Starling, *Sturnus vulgaris*) in the farm study was resistant to four antimicrobials, the isolate was resistant to two antimicrobial categories only and was not considered to be MDR. Except for three (3.4%) isolates obtained from the wildlife study that showed resistance to more than five antimicrobials, isolates obtained from both sources in the wildlife and farm studies demonstrated similar prevalence of resistance to all tested antimicrobials.

Relevant to our findings, Nesemeier., *et al*. [35] observed high AMR rates (58%) in *Salmonella* to at least one antimicrobial in a study on the prevalence of AMR in *Salmonella* shed from range and feedlot cattle. They reported that 42% of isolates tested were susceptible to all antimicrobials tested, which was higher than those (7.4%) of our findings (data not shown). In the study, they also found that 56% were resistant to two or more antimicrobials and 3% demonstrated resistance to only one antimicrobial (Ceftiofur). However, our study demonstrated overall 9.1% resistance to two or more antimicrobials and 21.5% resistance to a single antimicrobial only including AMP, CIP, NAL, STR, SXT, and TCY. This discrepancy may be due to the difference in antimicrobial panels used. It is also possible for *Salmonella* isolates to develop different degrees of resistance to antimicrobials depending upon their prior exposure to environments and type of their species.

The susceptible, intermediate, and resistant patterns of *Salmonella* isolates to the 12 antimicrobials tested in this study (Figure 2) revealed that TCY resistance was most common (16 isolates, 13.2%), followed by resistance to STR (12 isolates, 9.9%) and AMP (10 isolates, 8.3%). In addition, a majority (70 isolates) demonstrated intermediate resistance to STR. In their study, Nesemeier., *et al*. [35] reported that isolates obtained from cattle showed the most frequent resistance to CHL (57%), followed by TCY (56%), STR (56%), AMP (55%), and AMC (47%), while no resistance to AMK, CIP, GEN, NAL, or SXT was observed. In study with chicken meat, Tîrziu., *et al*. [45] found that *Salmonella* isolates showed resistance most frequently to TCY (66.6%), NAL (64.3%), CIP (61.9%), and STR (59.5%). In addition, other studies also reported high AMR rates in *Salmonella* isolates originating from chicken to TCY (56.2 - 87.1%) [34,48], NAL (50.0 - 98.8%) [2,6,34,47], CIP (42.1 - 59.4%) [34,47], and STR (64.5 - 86.1%) [34,48].

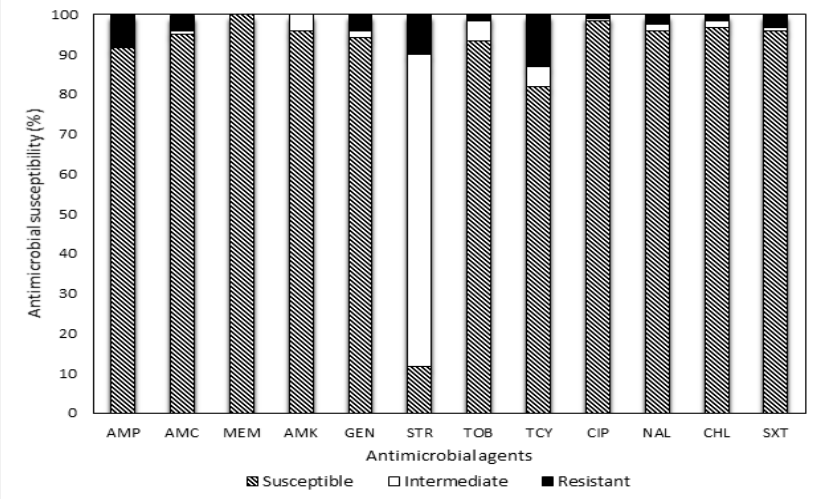

***Figure 2**: Prevalence of resistance to 12 antimicrobial agents in 121 Salmonella isolates.*

Tîrziu., *et al*. [45] speculated that their high resistance was attributed to the possible overuse of these antimicrobials in the poultry industry. In our study, all *Salmonella* isolates were susceptible to MEM, while susceptibility to AMC, AMK, CIP, NAL, CHL, and SXT was frequently observed at a rate of greater than 95%. More specifically, *Salmonella* isolates obtained from the farm study showed no resistance to a total of 8 antimicrobials (AMC, MEM, AMK, TOB, TCY, CIP, CHL, and SXT), while isolates from the wildlife study showed no resistance to only two antimicrobials (MEM and AMK), indicating a higher prevalence of resistance to multiple antimicrobials in *Salmonella* isolates obtained from wildlife. Based on the findings from our study, as well as those from others [2,6,34,35,45,47,48], a lack of resistance in *Salmonella* to MEM and AMK and very high susceptibility (> 95.0%) to SXT indicate the effectiveness of those antimicrobials in the treatment of *Salmonella* infections in veterinary and human medical practices. Previous studies that were similar to ours [23,25,35] looked at samples from cattle feces, hides and carcasses and reported 100% susceptibility to AMK and SXT in *Salmonella*. In addition, the limited results from the farm study also revealed no correlation of *Salmonella* isolates between AMR and PFGE profiles.

While 73 (93.6%) *Campylobacter* isolates were resistant to one or more antimicrobials (Table 2), only two (2.6%) isolates were susceptible to all tested antimicrobials, indicating 97.4% of *Campylobacter* isolates were non-susceptible to one or more antimicrobials (data not shown). MDR was observed in 18 (23.1%) isolates comprised of 88.9% sheep and 11.1% goat, which is in agreement with previous observations about *E. coli* and *Salmonella* that show higher prevalence in sheep than goats, as described previously.

STX resistance was most common among the *Campylobacter* isolates (42 isolates, 53.8%), followed by resistance to NAL (47.4%), and AMP (25.6%) (Figure 3). Susceptibility to GEN (73 isolates, 93.6%), MEM (72 isolates, 92.3%), CHL (69 isolates, 88.5%), AMC (68 isolates, 87.2%), CIP (68 isolates, 87.2%), and AMK (66 isolates, 84.6%) was frequently observed in *Campylobacter* isolates. Two strains of *Campylobacter* isolated from a fecal sample from sheep at the Virginia State University research farm were non-susceptible to 10 out of 12 antimicrobials tested in this study. Both were susceptible to TCY while each was susceptible to either MEM or CHL. According to a CDC study [11], resistance to CIP in *C. jejuni* isolates obtained from human cases of infection has continuously increased from 12% in 1997, 21% in 2002, and 26% in 2007, whereas no isolates in the US were resistant to CIP in 1989 and 1990.

More recently, the European Food Safety Authority [20,21] reported even higher resistance (35.8% - 69.8%) to CIP in *C. jejuni* isolates from both humans and animals from its Member States. Another study [39] conducted in Spain also found extremely high CIP resistance rates in *Campylobacter* strains isolated from broilers (99%), pigs (100%), and human feces (72%), along with high cross-resistance rates between CIP and NAL. In contrast to their findings, a low level of resistance (5.1%) with additional 7.7% intermediate to CIP and 64.1%

non-susceptibility to NAL were shown in our study. Sáenz., *et al.* [39] indicated that the use of quinolones in veterinary practices mostly attributed to the increase in CIP resistance. The present survey revealed that the prevalence of non-susceptibility to 12 antimicrobials tested in the current study was the highest in E. coli (41.0%), followed by Campylobacter (25.9%), and then Salmonella (12.5%). Among all the tested antimicrobials, TCY showed the highest frequency of resistance among *E. coli* (62.1%) and Salmonella (13.2%) isolates, while *Campylobacter* (53.8%) was most resistant to SXT demonstrating different resistance patterns among the bacteria in this study. The most effective antimicrobials tested in this study are CIP for E. coli, MEM for *Salmonella*, and GEN for *Campylobacter*. Of all 265 isolates evaluated, approximately 62% showed resistance to at least one antimicrobial tested. Additionally, susceptibility of microorganisms to antimicrobials was not PFGE specific. However, in contrast to our findings that none of the PFGE profiles in each type of bacteria demonstrated any correlation with their prevalence to antimicrobials (data not shown), a prior study done by Zhao., *et al.* [50] showed a good correlation of bacterial PFGE profiles with their AMR profiles.

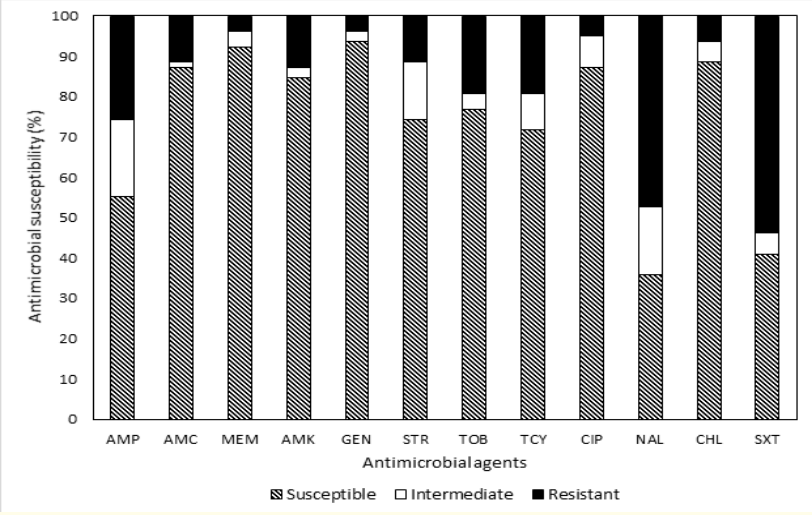

Figure 3: *Prevalence of resistance to 12 antimicrobial agents in 78 Campylobacter isolates.*

The results from the present study, though from a limited geographical region, show a level of consistency between the prevalence of resistance to certain antimicrobial categories evaluated for this study and their usage levels in the US from 2000 and 2010 [12]. Nationally, penicillins and tetracyclines are two of the most commonly used antimicrobial categories. The isolates of *E. coli* and *Salmonella* evaluated in the present study consistently showed the highest prevalence of resistance to these antimicrobial categories. These isolates showed most resistance to the tetracyclines, then to either penicillins or aminoglycosides. In contrast, *Campylobacter* isolates demonstrated a distinct pattern of resistance. These isolates showed the highest prevalence of resistance to folate pathway inhibitors, which were followed by quinolones and penicillins.

Conclusion

In conclusion, findings from the present study confirm the high prevalence of broad-spectrum AMR in bacteria in the environment re-emphasizing the need for judicious and careful use of antimicrobials in human and agricultural practices in an effort to reduce future manifestations of MDR bacteria in foodborne illnesses. The findings may benefit the NARMS by providing data of AMR surveillance in food samples and in farm-reared livestock and wildlife, further assisting in the development of new antimicrobials and promoting interventions to reduce resistance among foodborne bacteria. Continued research and efforts on a large scale are needed to provide a better understanding of AMR in bacteria in relation to the environment and genomic relatedness among bacterial species.

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