Surveillance for antibiotic-resistant *E.coli* in wildlife feces from areas disturbed by agricultural and residential land uses in Appalachian Kentucky

Olivia S. Salazar, B.S Environmental Science Student; Jason W. Marion, Ph.D., Faculty Mentor

Department of Natural Sciences , The University of Virginia at Wise, Wise, VA, USA

Department of Environment Health Sciences, Eastern Kentucky University, Richmond, KY, USA

INTRODUCTION

When exposed to antibiotics, bacteria can develop antimicrobial resistant genes (AMR) via processes such as horizontal gene transfer between species and genetic transfer mechanisms of transformation, transduction, and conjugation[2]. Bacteria are subject to these antibiotics and therefore vulnerable to AMRs due to the use of antibiotics by humans over extended periods in clinical, pharmaceutical, and agricultural environments for treatments of diseases. Misusing these antibiotics has led to a rapid increase in AMRs worldwide, leading to a critical global threat, according to several health organizations such as World Health Organization (WHO)[2]. Some bacteria may acquire AMR to multiple antibiotic classes, leading to increased multidrug resistance (MDR) persistence in bacterial populations, particularly potential pathogens, further adding to the complexity of this life-threatening global issue[1].Wild animals have the potential to be reservoirs of resistant bacteria and disseminate the bacteria throughout the environment due to their close association with humans. Some studies have found resistance in birds, reptiles, mammals, and fish[1]. In this study, we determined if there was a correlation between types of land use and rates of antimicrobial-resistant *E.coli* in wildlife. *E.coli* was chosen as the tested bacteria because it is found in many animals with resistant genes in fecal samples. It is ideal for the study of the dispersal of antimicrobial resistance across diverse vertebrate host species and the environment[3]. We looked at developed, agricultural, and forested areas. A wide variety of animal fecal samples were collected using a convenience collection sampling method known as roadkill. In addition, we are looking to see if there was a difference in the antimicrobial-resistant *E.coli* found in mammals and those found in avian animals.

METHODS

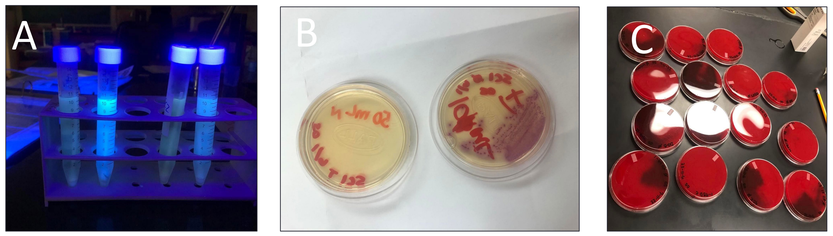
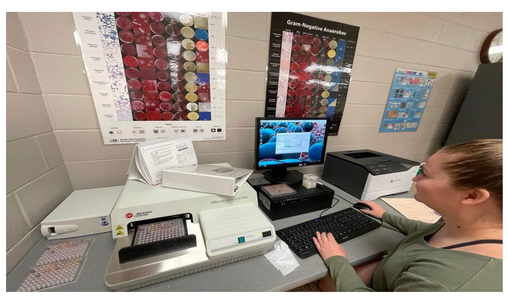


Figure 1: Microbiological media used in this study; (A) Coliglow media prepared in vials, (B) two modified mTEC plates, (C) blood agar plates.

- Convenience sample collection: primarily roadkill (n = 31) and manure/scat (n = 20)
- Most roadkill obtained from Kentucky's roadways in Eastern & Central Kentucky.
- For forested and agricultural areas, rural roads and highways were used.
- Residential areas provided samples in parts of Madison and Clark Counties.
- Samples were identified as mammalian or avian.
- Three sterile swabs were used to swab the anal cavity of the animal or, if not prominent, the nearest opening. The three swabs were then placed in one of three ColiGlow broth media tubes: (1) no antibiotic, (2) tetracycline treated, and ciprofloxacin-treated).

^ Project pics ☺



- Upon incubation at 35°C for 24h, tubes were assessed for fluorescence using a Spectroline UV lightbox at 254 nm. Fluorescing tubes were presumed *E. coli* positive. Fifty-one samples were collected from various animals, including raccoons, opossums, black bears, skunks, geese, cardinals, and crows.
- Presumed *E. coli* positive samples were further assessed for microbial ID and antibiotic susceptibility/resistance profiling using the Microscan (left)

- Isolates were obtained by streak plating one loopful of positive broth onto modified thermotolerant *E. coli* agar (mTEC) plates and incubating at 35°C for 2h and 44.5°C for 22h. Isolates obtained from mTEC plates were then placed on general media (blood agar) for loading the Microscan® Urine Panel.
- Of the 51 samples, 31 were run through additional analyses using the Microscan autoSCAN4 to test antibiotic resistance to ciprofloxacin, tetracycline and 23 other antibiotics and to do microbial ID.

RESULTS & DISCUSSION

Table 1: Presence/absence of tetracycline and ciprofloxacin-resistant *E.coli* in avian and mammal species.

ColiGlow Media Antibiotic Used	Positive/n (%)	Avian Positive/n (%)	Mammal Positive/n (%)	Avian vs Mammal Exact *p*
Tetracycline	30/41 (73%)	1/5 (20%)	29/36 (81)	***0.014***
Ciprofloxacin	23/41 (56%)	1/5 (20)	22/36 (61)	0.15

Table 2: Microscan results of 31 isolates providing microbial ID and level of antibiotic resistance. R = resistant, S = susceptible, I = intermediate

Sample ID	Media	Organism	CP	TE
BBRS_0*	No Treatment	*E.coli*	S	R
WPSI_0*	No Treatment	*E.coli*	S	S
US-25OP_0*	No Treatment	*E.coli*	S	S
DAISY(90R_0)	No Treatment	*P.mirabilis*	S	R
90R2_0*	No Treatment	*K.oxytoca*	S	S
DGB_0*	No Treatment	*S.marcescens*	S	R
BCO_0*	No Treatment	*E.cloacae*	S	S
1-64OP_0	No Treatment	*K.ozaenae*	S	R
LCW4_0*	No Treatment	*S.liquefac cplx*	S	R
LC2_0*	No Treatment	*K.intermedia*	S	R
89O_0*	No Treatment	*K.pneumoniae*	S	R
QR_0	No Treatment	*S.odorifera*	S	I
MWGH_0*	No Treatment	*E.cloacae*	S	S
CEBB_0*	No Treatment	*E.cloacae*	S	S
519R_0*	No Treatment	*K.pneumoniae*	S	R
EKUS4T_1	Tet-Treatment	*A.caviae cplx*	S	R
EKUF7T_1	Tet-Treatment	*E.coli*	R	R
QR_T	Tet-Treatment	*E.coli*	S	R
BBRS_T	Tet-Treatment	*E.coli*	S	R
EKUS4T_2	Tet-Treatment	*E.coli*	S	R
EKUF9T_2	Tet-Treatment	*E.coli*	S	R
EKUF9T_1	Tet-Treatment	*E.coli*	S	R
EKUF7T_2	Tet-Treatment	*E.coli*	S	R
DGBT_O-*	Tet-Treatment	*Y.pseudotb*	S	I
EKUF3C_I	Cip-Treatment	*E.coli*	R	R
EKUF8C_I	Cip-Treatment	*E.coli*	R	R
EKUF8C_2	Cip-Treatment	*E.coli*	R	R
EKUF3C_2	Cip-Treatment	*E.coli*	R	R
EKUF10C_2	Cip-Treatment	*E.coli*	R	R
EKUF10C_1	Cip-Treatment	*E.coli*	R	S
89O_C*	Cip-Treatment	*E.coli*	S	S
GLADIE_C*	Cip-Treatment	*E.coli*	S	S

Table 3: Percentage of land uses in relation to collection locations.

Combined Land Use	Mean (%)	SD	Median (%)	Range
Agricultural	47.67	31.39	51.60	0.06 - 79.06
Forested	33.29	33.57	11.06	1.12 - 98.89
Developed	18.04	19.53	8.79	0.74 - 91.75

Table 4: Resistant *E. coli* frequencies by agricultural intensities.

Ag Intensity	n	Mean (%Ag)	Median (%Ag)	Range (%Ag) low – high	tetracycline resistant n (%)	ciprofloxacin resistant n (%)
low	9	5.78	1.02	0.06-19.52	6/9 (67)	5/9 (56)
medium	11	48.7	50.96	36.41-64.25	3/11 (27)	2/11 (18)
high	21	78.95	78.95	73.27-79.06	21/21 (100)	16/21 (76)
Exact test *p*					***<0.001***	***0.008***

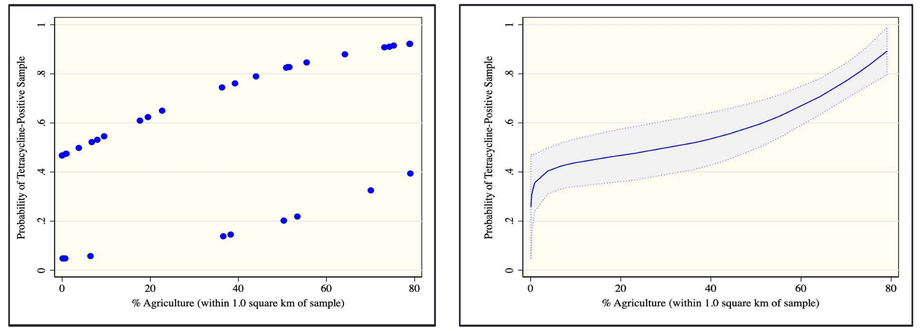


Figure 2: Scatter (left) of logit-model predicted probability of observing tet-positive *E. coli* in a sample as a function of %Agriculture for avian and mammal samples, with lowess-smoothed results w/ the 95% prediction interval.

Covariate	Beta	aOR (95% CI)	*p*
Mammal vs. Avian	2.904	18.25 (1.51 - 221)	***0.023***
%Agriculture	0.033	1.03 (1.01 - 1.06)	***0.020***
Constant Term	-3.047		

Overall Recovery of *E. coli*: Of the 51 samples collected, 41 (80%) were *E.coli* positive. Among mammal samples, 36/39 (92%) were from positive for *E. coli.* Among avian samples, 5/11 (45%) were *E. coli* positive. For antibiotic resistance, 30/51 (59%) were tetracycline-positive and 23/51 (56%) were ciprofloxacin-positive. **Table 1** illustrates results for when *E. coli* were successfully recovered in the ColiGlow media. In these samples, **Table 1** demonstrates that tetracycline resistance was common (81% positive) in mammals, but not avian samples (20% positive). Similarly, ciprofloxacin resistance was common (61% positive) in mammals, but not avian samples (20% positive).

Microscan Results: For assessing resistance, among tetracycline-treated ColiGlow media isolates, 8 of 9 (88.9%) were resistant and 1/9 (11.1%) was intermediately resistant. Among eight Ciprofloxacin-treated ColiGlow media isolates , six (75%) were resistant. These results indicate the screening method is mostly accurate for resistance determination. In terms of microbial ID, in the untreated media, recovery of competing (non-*E. coli*) organisms was quite common (**Table 2**).

Land Use Relationships to Resistant Bacteria: Percent land uses within 1.0 km^2 were obtained from the area surrounding each collection point using ArcGIS and the National Land Cover Database. Land uses were categorized into three categories (**Table 3**). For assessing the presence/absence of positive samples for resistance, logistic regression indicated only one significant relationship; specifically, tetracycline-resistant bacteria with %agriculture (crude odds ratio = 1.03; 95% CI: 1.01 – 1.06). **Table 4** further confirms this positive association using Fisher Exact Test, whereby increasing agricultural intensity (>73%) was associated with the most tet- and cipro-resistant bacteria.

Multivariable Analysis: Given the impact of mammal vs. avian samples, multivariable logistic regression was used to account for sample type, and upon adjusting for significant mammal carriage of tet-resistant *E. coli*, a 3% increased odds for observing a tet-resistant sample is expected for each 1% increase in percent agriculture (cultivated crops and/or hay/pasture) illustrated in Figure 2. The model accurately predicts 86.97% of tet-positive samples using the area under the ROC curve in Stata 15.

Conclusions: There have been few studies that examine the presence of antimicrobial resistance across a wild variety of animals that live in close proximity to urban environments and even fewer that have compared observed resistance relative to developed, agricultural, and forested areas in Appalachian Kentucky. Wildlife species provide a valuable insight into how resistance can be gained and transferred in ecosystems. In this study, we found the percentage of antimicrobial resistance was positively correlated with agricultural land uses. This could be due to an influx of antibiotics being distributed to livestock and subsequent manure spreading on fields. Further studies are needed as these results were limited by sample size and non-random convenience sampling. Despite these limitations, these results add to the literature and evidence suggesting a need to consider ecosystem approaches for informing the epidemiology of antimicrobial resistance (One Health Approach).

ACKNOWLEDGEMENTS / REFERENCES

Funding for this project was provided by the National Science Foundation Research Experience for Undergraduates Program (PI: Brown, CoPI: Watson; Award #1950355). Great appreciation is extended to Dr. Jason Marion and the Department of EHS and MLS for seeking resources and all the technical advice on this project. I am thankful for Dr. Travis Altheide at EKU for the lab assistance with the Microscan© and other lab equipment. Lastly, I would like to thank Joshua Castle for the assistance with the GIS work to satisfy our land usage data as well Jayden Hamlet for help in the field.

1. Jardine, C. M.; Janecko, N.; Allan, M.; Boerlin, P.; Chalmers, G.; Kozak, G.; McEwen, S. A.; Reid-Smith, R. J. Antimicrobial resistance in escherichia coli isolates from raccoons (Procyon lotor) in southern Ontario, Canada. Applied and Environmental Microbiology 2012, 78, 3873–3879.
2. Mukherjee, M.; Marie, L.;Liles, C.; Mustafa, N.; Bullerjahn, G.;Gentry, T.J.; Brooks, J.P. Elevated Incidences of Antimicrobial Resistance and Multidrug Resistance in the Maumee River (Ohio, USA), a Major Tributary of Lake Erie. Microorganisms 2021, 9, 911.https://doi.org/10.3390/microorganisms9050911
3. Hassell, J. M.; Ward, M. J.; Muloi, D.; Bettridge, J. M.; Robinson, T. P.; Kariuki, S.; Ogendo, A.; Kiiru, J.; Imboma, T.; Kang'ethe, E. K.; Öghren, E. M.; Williams, N. J.; Begon, M.; Woolhouse, M. E.; Fèvre, E. M. Clinically relevant antimicrobial resistance at the wildlife–livestock–human interface in Nairobi: An epidemiological study. *The Lancet Planetary Health* **2019**, *3*.