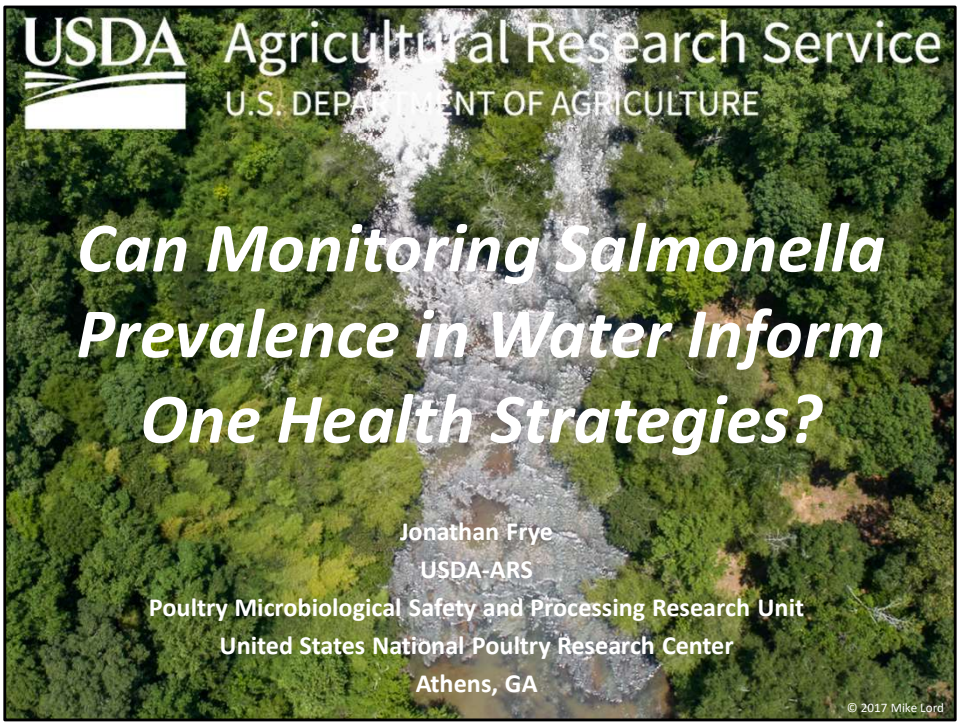
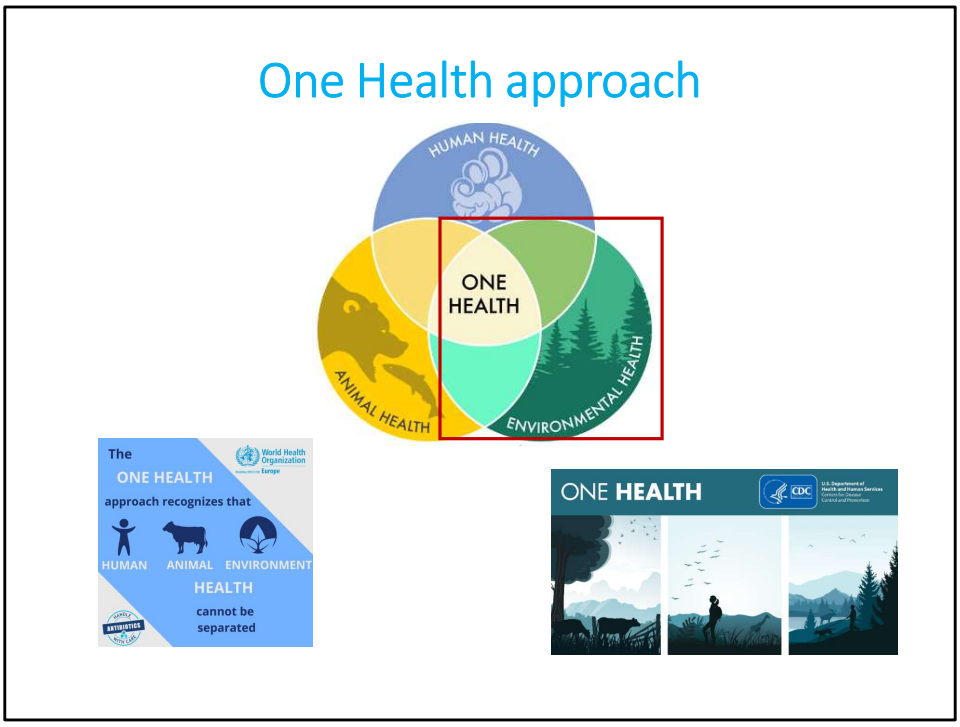


Good morning, I would like to thank IAFP for inviting me to present our work today.

Outline

- What is One Health
- Surveillance of multiple components of antimicrobial resistance (AR) in a watershed
  - Isolate bacteria and assay for AR and AR genes
  - qPCR of AR genes and source tracking markers
  - Quantitation of 26 antibiotics
- Results
- Conclusions
- How does this information effect the One Health approach to antimicrobial resistance?



Today, I am going to talk about the research completed by Gabi Cho during her PhD and postdoc years, which is on the bacteria found in the environment, particularly surface water.

So, why am I interested in water? Why am I interested in the environment?
One Health approach states that the health of humans and animals are connected to the health of environment, and we have to work together in order to understand all three compartments and thereby achieving optimal health outcomes for all. This One health approach is also used to tackle the global problem of AR. However, the environment was a big data gap compared to human and animals, and we decided to study bacteria present in the water environment, especially pathogenic bacteria and AR bacteria, to be able to see the whole picture of what is going on.

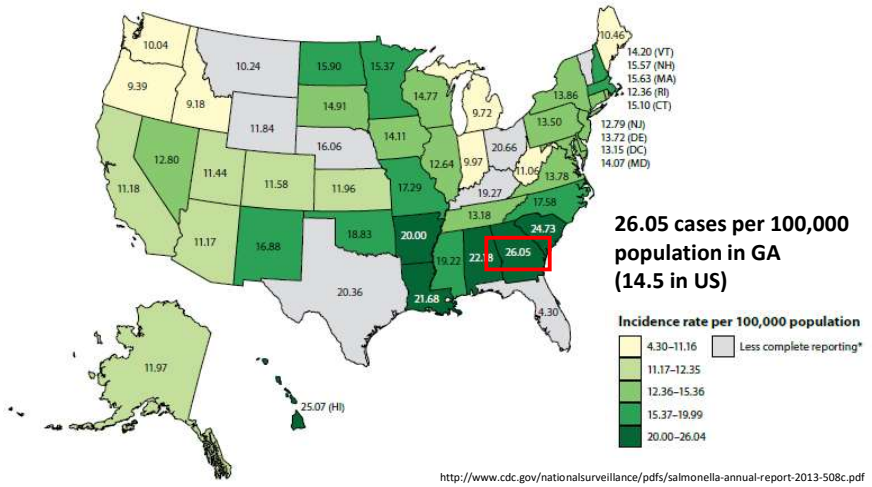
Background to the Study

- **Gabi Cho student rotation in BEAR, Fall 2014**
  - **Interested in water safety due to her experience as a child of a missionary family in India**
  - **We don't do water. But… Meinersmann *et al.* 2006**
  - **Lead SY and NPL approve the study**
- **One Health approach to AR is needed**
  - **Collaboration with the Upper Oconee Watershed Network (UWON) and UGA**
  - **Winter 2015 begin quarterly sampling**
  - **Winter 2020 was last sampling due to the pandemic**

Sohyun Cho, or Gabi was a new graduate student and was interested in studying antimicrobial resistant bacteria in water because when her family were missionaries in India, she saw her friends and family struggle to get safe water and often got sick from the water.
I said we don't do water, but I remember we had done water with Rick Meinersmann and that the our local watershed had high levels of bacteria and AR.
We showed this to our lead scientist, Charlene Jackson, and she said the environment was a big data gap, so we presented this to our NPL James Lindsey, and he liked the idea and approved the research because the one health approach to AR required data from the environment.
So we developed a collaboration with Erin Lipp and Elizabeth Ottesen at UGA and with the upper Oconee watershed network volunteers.

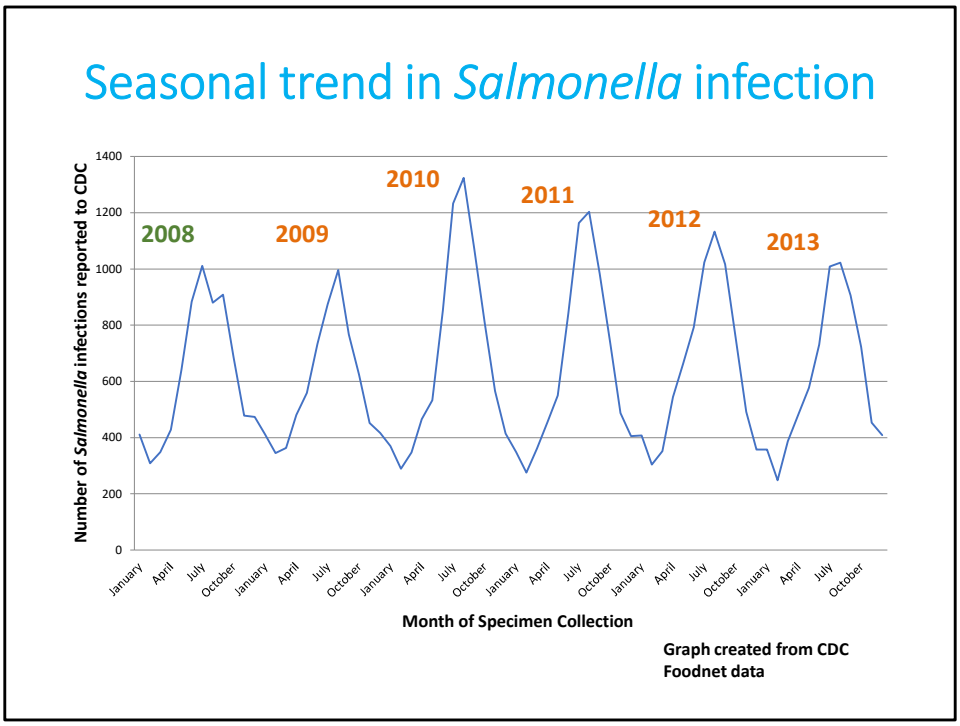
Geographical trend in *Salmonella* infections

Incidence rate of laboratory-confirmed human *Salmonella* infection reported to CDC by reporting jurisdiction, 2013 (n= 45,735)

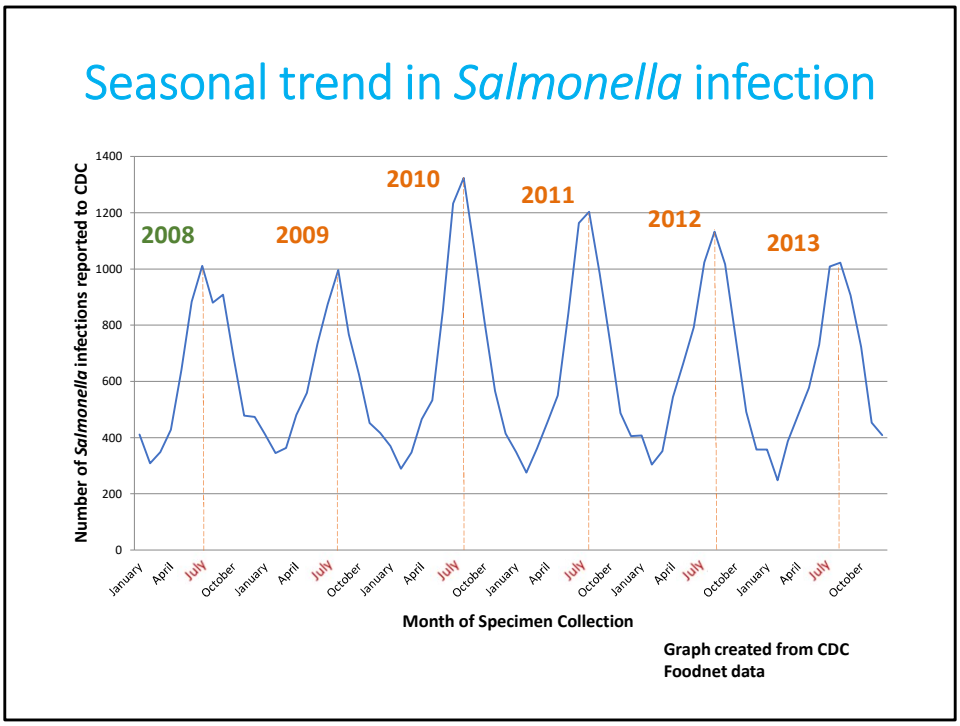


http://www.cdc.gov/nationalsurveillance/pdfs/salmonella-annual-report-2013-508c.pdf

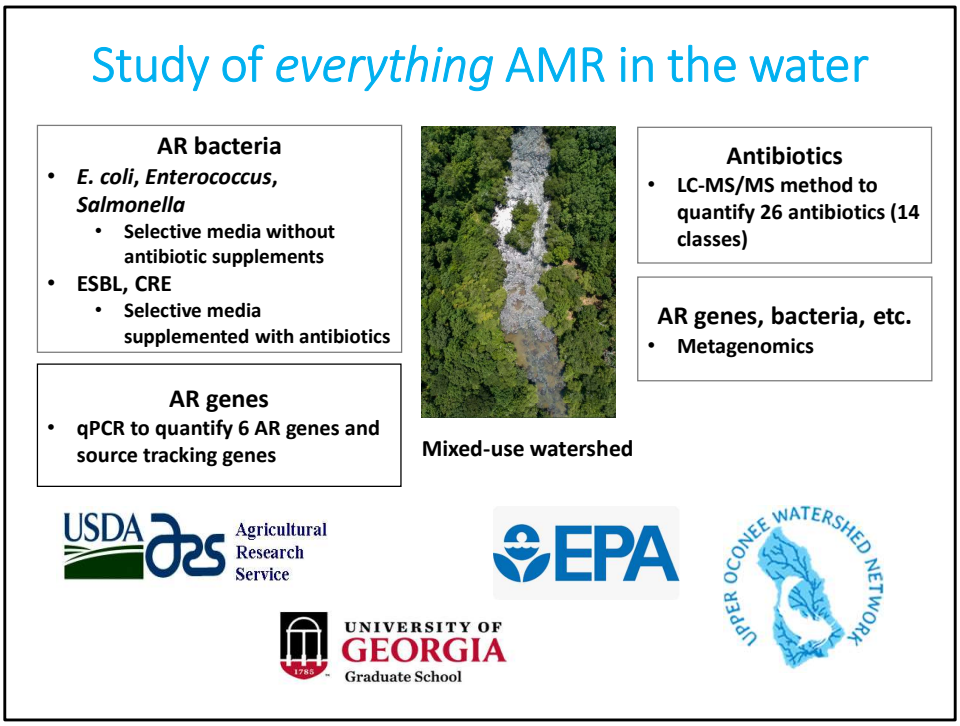
This map shows that Salmonella infection is highest in Southeastern US as depicted by the dark green color.
In 2013, Georgia had the highest case rate of Salmonella infections with 26.05 cases per 100,000 compared to the US average of 14.5.



This graph shows a seasonal trend in Salmonella infections. Y-axis represents the number of Salmonella infections reported to CDC and X-axis represents the month the specimens were collected. There is a clear pattern repeating every year with more infections during summer months.



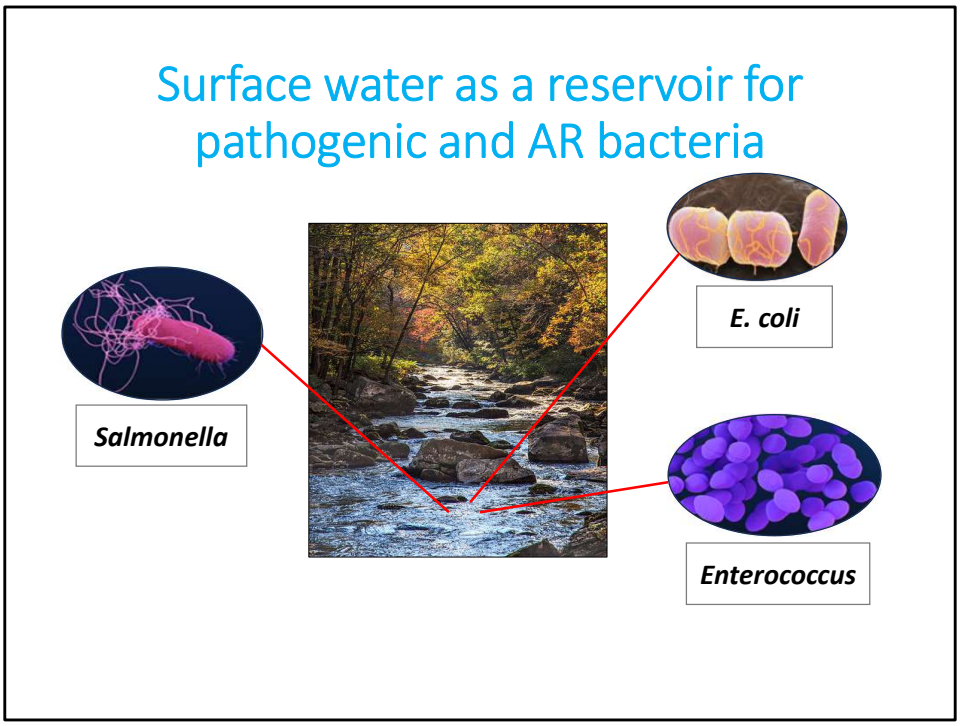
This graph shows a seasonal trend in Salmonella infections.
Y-axis represents the number of Salmonella infections reported to CDC and X-axis represents the month the specimens were collected.
There is a clear pattern repeating every year with more infections during summer months.
Why would a food borne disease be seasonal?
There simply can't be that much potato salad, plus, most cases reported are not associated with an outbreak and are what we call sporadic cases with no known source.
So what are some other reasons summer months it goes from less than 400 to nearly three times that?
Other things people do in the warm months is outdoor recreation, and a lot of that has contact with surface water, including swimming, fishing, boating, etc.
So, why not look for the cause of this increase in the environment?



We not only isolated ARB from surface water to characterize the bacteria that are culturable and express their ARGs, but also quantified the total ARGs present within the whole bacterial populations in the watershed, including those that have not been expressed.

In addition, antibiotics that are important to human and veterinary medicine were measured to investigate the occurrence and distribution of these antibiotics in aquatic environments.

As WWTPs have been proposed to be the hotspots for the emergence of ARB and from where these bacteria are spread into the natural environment, influents and effluents from three WWTPs located within the watershed and whose effluents flow into the streams within the watershed were included in the analyses.



As you all know, Salmonella are pathogenic bacteria and while E. coli and Enterococcus are commensal bacteria, certain E. coli, such as O157:H7, and E. faecalis and E. faecium, are pathogenic and can infect humans and animals. Also, these bacteria were chosen by NARMS or National Antimicrobial Resistance Monitoring System as sentinel organisms for monitoring AR in food animals, retail meats, and humans, so we chose to study these bacteria to monitor AR in surface water as well.

Surface water receives contamination from the surroundings and human and animals can be exposed to contaminants in the water through recreational activities, drinking, and consuming fruits and vegetables irrigated with contaminated water. So the question I had was- Is surface water a reservoir of pathogenic and AR bacteria that can be transmitted to or from humans and animals?

Experimental Design: Sampling

- **Collaboration with the Upper Oconee Watershed Network, UOWN**
- **105 sites chosen to represent different land uses**
- **Sites are sampled each quarter by UOWN citizen scientist volunteers who collect 1L from each site in a sterile bottle**
- **Number of sites sampled determined by number of volunteers**
  - **A core set of 40 sites usually tested**
  - **All sites tested in Spring "River Rendezvous" event**
- **Samples kept at 4°C until analyzed**

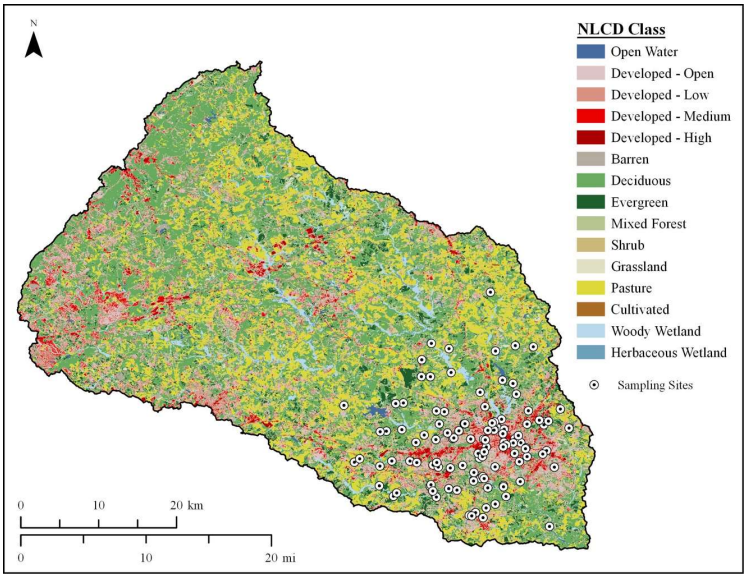
USDA

UPPER OCONEE WATERSHED NETWORK

Department of Microbiology
Franklin College of Arts and Sciences
UNIVERSITY OF GEORGIA

Read slide

Experimental Design: UOWN sampling of the Upper Oconee Watershed

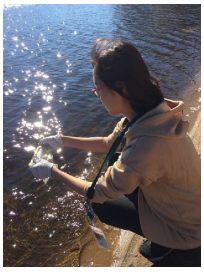


This map shows the entire Upper Oconee Watershed, with land use shown in these colors, and the sampling sites shown as black and white circles.
The waterways we sample merge to form the Oconee River which then joins the Altamaha River which flows into the Atlantic Ocean south of Sapelo Sound.

Sampling: The Upper Oconee watershed

UOWN volunteers collecting water samples

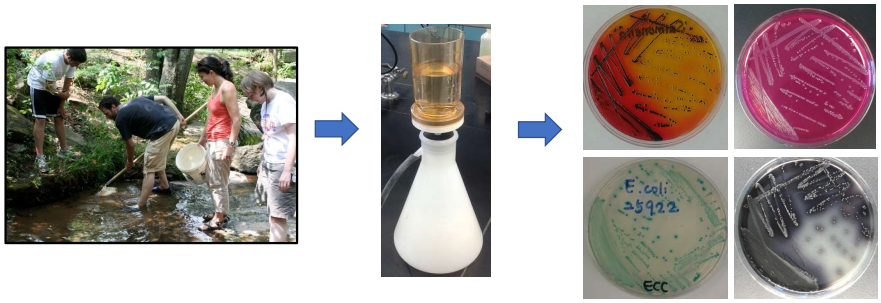
1L water bottle



UWON volunteers. Ahn Nguyen. Gabi. 1 liter bottle.

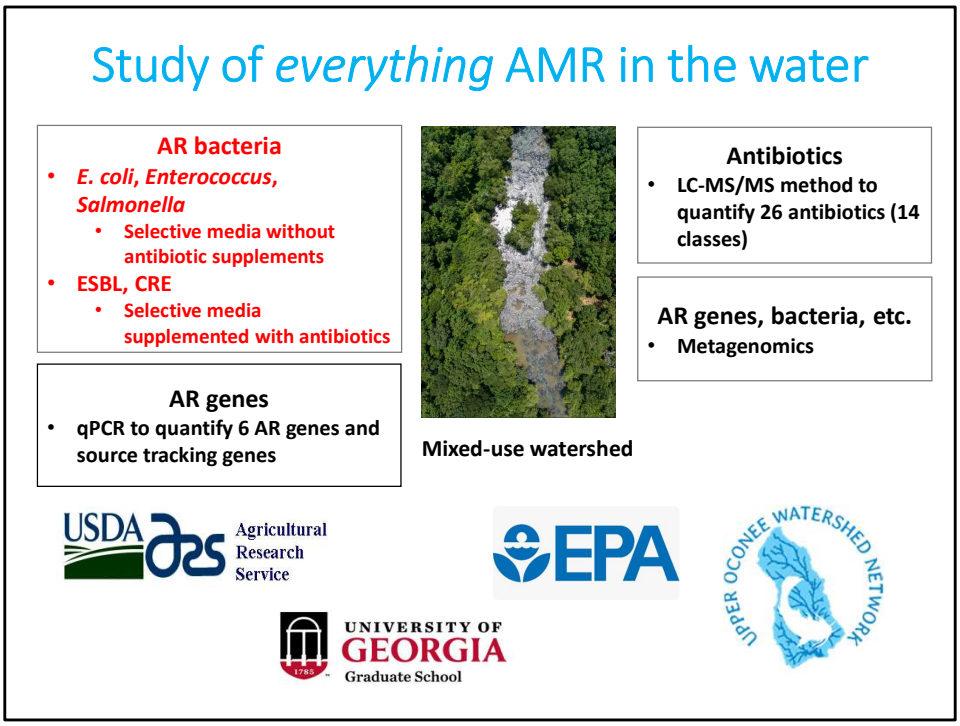
Processing

Isolation of *Salmonella, E. coli,* and *Enterococcus* and their phenotypic and genotypic characterization



Sampling Filtration Isolation

We got about 30 to 100 water samples for each water collection four times a year, depending on the number of volunteers to help us collect water samples. Some of these sites were adjacent to animal farms, wastewater treatment plants, and residential areas with septic tanks.
We filtered the water samples and isolated Salmonella, E. coli and Enterococcus using selective media.



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In addition, antibiotics that are important to human and veterinary medicine were measured to investigate the occurrence and distribution of these antibiotics in aquatic environments.

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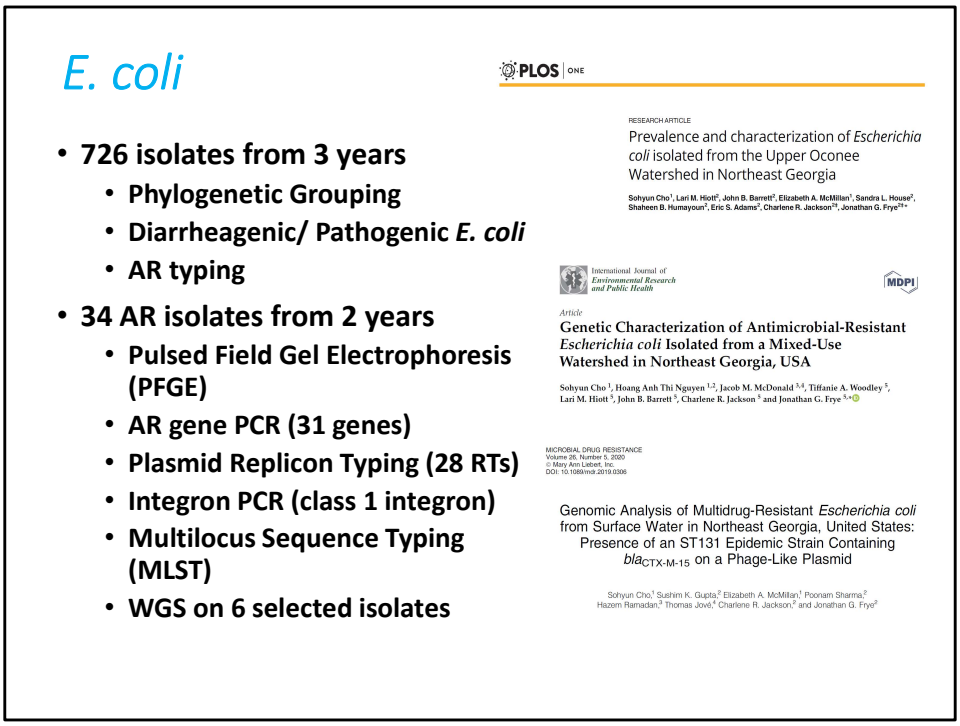
Results

sampling season	number of samples	% of positive sites (no. of isolates recovered)		
		Salmonella	*E. coli*	*Enterococcus*
Winter 2015	30	70.0 (59)*	96.7 (56)*	96.7 (58)*
Spring 2015	100	68.0 (153)*	99.0 (99)	93.0 (93)
Summer 2015	33	81.8 (66)*	97.0 (46)*	100.0 (196)*
Fall 2015	59	30.5 (37)*	100.0 (59)	98.3 (58)
Winter 2				(41)
Spring 2				(87)
Summer 2				(27)
Fall 201				(77)
Winter 2				(51)
Spring 2				(94)
Summer 2				(40)
Fall 201				(43)
Winter 2018	41	48.8 (57)*	100 (41)	97.6 (40)
Spring 2018	42	59.5 (74)*	100 (42)	100 (42)
Summer 2018	44	93.2 (94)*	100 (44)	100 (44)
Fall 2018	44	61.4 (55)*	97.7 (43)	100 (44)
Winter 2019	44	59.1 (71)*	100 (44)	100 (44)
Spring 2019	41	78.0 (99)*	100 (41)	100 (41)
Summer 2019	19	89.5 (40)*	100 (19)	100 (19)
Fall 2019	45	60.0 (64)*	100 (45)	100 (45)
Winter 2020	44	63.6 (52)*	100 (44)	100 (44)

1,052 water samples
70.1% positive for *Salmonella* (n = 1,796)
99.5% positive for *E. coli* (n = 1,103)
98.9% positive for *Enterococcus* (n = 1,228)

* More than one isolate obtained per site due to the use of several media

Now let's go over the results. This is the result of the 21 water collections we have had until we had to stop due to COVID pandemic.
A total of 1,052 water samples were collected, and almost all of the water samples were positive for E. coli and Enterococcus, and about 70% of all the water samples were positive for Salmonella. More than a thousand isolates were recovered for each of the bacteria, but due to limited time we have today, I am going to talk about just a few selected isolates that might be of interest.



For E. coli, I took my 750 E. coli isolates from the first 3 years of my study and determined their phylogenetic groups, pathogenic types, and AR using the methods mentioned earlier. After that, I selected 34 AR E. coli isolates to run PFGE, AR gene PCR, plasmid replicon typing, integron PCR, MLST, and WGS for 6 selected isolates.

And these are the papers that came out of these data:

-Prevalence and characterization of *Escherichia coli* isolated from the Upper Oconee Watershed in Northeast Georgia

-Genetic Characterization of Antimicrobial-Resistant *Escherichia coli* Isolated from a Mixed-Use Watershed in Northeast Georgia, USA

-Genomic Analysis of Multidrug-Resistant *Escherichia coli* from Surface Water in Northeast Georgia, United States: Presence of an ST131 Epidemic Strain Containing *bla*CTX-M-15 on a Phage-Like Plasmid

Enterococcus

- **865 isolates from 3 years**
  - ***Enterococcus* speciation**
  - **AR typing**
- **51 MDR (≥3 AR) isolates from 2 years**
  - **AR gene PCR (27 genes)**
  - **Plasmid Replicon typing (21 RTs)**
- **WGS on daptomycin (n=12) and tigecycline (n=20) resistant isolates**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ORIGINAL ARTICLE

Diversity and antimicrobial resistance of *Enterococcus* from the Upper Oconee Watershed, Georgia

S. Cho[1], L.M. Hiott[2], J.M. McDonald[3], J.B. Barrett[2], E.A. McMillan[1], S.L. House[2], E.S. Adams[2], J.G. Frye[2] and C.R. Jackson[2]

microorganisms

MDPI

Article

Antimicrobial Resistance Gene Detection and Plasmid Typing Among Multidrug Resistant Enterococci Isolated from Freshwater Environment

Sohyun Cho, John B. Barrett, Jonathan G. Frye and Charlene R. Jackson *

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

ELSEVIER

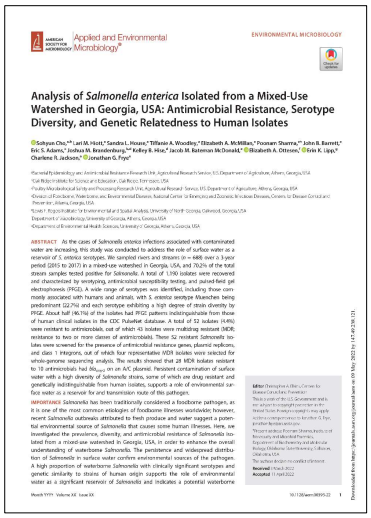
Evaluation of a new chromogenic agar for the detection of environmental *Enterococcus*

Sohyun Cho[1], Lari M. Hiott[1], Tiffanie A. Woodley, Jonathan G. Frye, Charlene R. Jackson*

Now changing to Enterococcus; I took 865 isolates from the first 3 years of my study and determined their species and AR phenotypes. And then, I have selected 51 MDR Enterococcus isolates from the first 2 years that are resistant to 3 or more antimicrobial drugs to run AR gene PCR and plasmid replicon typing.
Also, 32 isolates that were resistant to daptomycin and tigecycline, which are fairly new drugs, were selected for WGS.
And these are the papers that came out of these data:
-Diversity and antimicrobial resistance of *Enterococcus* from the Upper Oconee Watershed, Georgia
-Antimicrobial Resistance Gene Detection and Plasmid Typing Among Multidrug Resistant Enterococci Isolated from Freshwater Environment

Salmonella

- **1,190 isolates from 3 years**
  - **Serotyping**
  - **PFGE**
  - **AR typing**
- **52 AR isolates from 3 years**
  - **AR gene PCR (31 genes)**
  - **Plasmid replicon typing (28 RTs)**
  - **Integron PCR (class 1 integron)**
  - **WGS on 4 selected isolates**



Applied and Environmental Microbiology

ENVIRONMENTAL MICROBIOLOGY

Analysis of *Salmonella enterica* Isolated from a Mixed-Use Watershed in Georgia, USA: Antimicrobial Resistance, Serotype Diversity, and Genetic Relatedness to Human Isolates

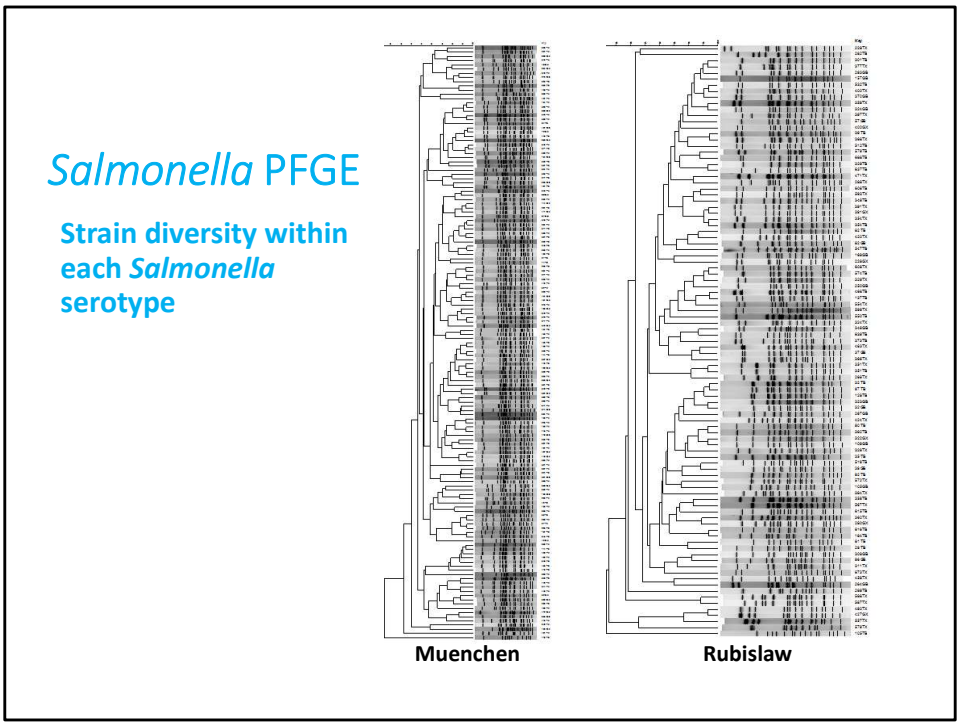
Next is Salmonella. I took my 1190 isolates from the first 3 years of the study and determined their serotypes, PFGE patterns, and AR. And then, I selected 52 AR Salmonella isolates to run AR gene PCR, plasmid replicon typing, integron PCR, and WGS for 4 MDR isolates.

And this is the paper that came out of these data: Analysis of *Salmonella enterica* Isolated from a Mixed-Use Watershed in Georgia, USA: Antimicrobial Resistance, Serotype Diversity, and Genetic Relatedness to Human Isolates.

Salmonella serotype

Agbeni	1	Havana	3	Newport	96
Agona	1	I 4,[5],12:b:-	53	Oranienburg	32
Anatum	36	I 4,[5],12:i:-	3	Orion	4
Aqua	7	Infantis	30	Ouakam	1
Baildon	2	Inverness	5	Paratyphi_B_var._L-tartrate+	1
Bareilly	29	Javiana	3	Rubislaw	153
Berta	3	Kentucky	5	Saintpaul	4
Braenderup	39	Kiambu	3	Schwarzengrund	9
Brandenburg	1	Litchfield	4	Senftenberg	4
Brazil	1	Liverpool	2	Soerenga	5
Cerro	2	Luciana	2	Tennessee	2
Cubana	10	Mbandaka	9	Thompson	4
Derby	1	Meleagidis	2	Typhimurium	19
Enteritidis	1	Mississippi	7	Worthington	1
Gaminara	25	Montevideo	113	Untypable	1
Give/ Give var.	57	Muenchen	270	*Salmonella* arizonae/ diarizonae	19
Hartford	87	Muenster	16	*Salmonella* houtenae	2
	Total			**1190**	

These are all the Salmonella serotypes we obtained from surface water with the number of isolates for each serotype, and the 5 most common serotypes we see are Hartford, Montevideo, Muenchen, Newport, and Rubislaw. You can see that some of the serotypes found in clinical isolates are also found in water, including Enteritidis, Infantis, Typhimurium, and Newport.



These are the PFGE patterns of the 2 most common serotypes, Muenchen and Rubislaw. This shows that each Salmonella serotype presents a high degree of strain diversity.
I compared my environmental isolates with human isolates on CDC PulseNet database and about half of my isolates had indistinguishable PFGE patterns as human clinical isolates, which means they could be clones. There were several incidences where the same *Salmonella* strains with the same PFGE patterns were simultaneously recovered from both surface water and humans in the surrounding area, suggesting a potential epidemiologic association between the aquatic environment and human infections.

Strain diversity of *Salmonella enterica* subspecies enterica serotypes

Serotype	total no. of isolates	% of total isolates	no. of samplings recovered	no. of PFGE patterns
Muenchen	270	22.7%	11	141
Rubislaw	153	12.9%	12	97
Montevideo	113	9.5%	11	31
Newport	92	7.7%	10	31
Hartford	87	7.3%	11	20
Give	57	4.8%	8	31
I 4,[5],12:b:-	53	4.5%	9	17
Braenderup	39	3.3%	7	11
Anatum	36	3.0%	5	7
Infantis	34	2.9%	8	13
Oranienburg	32	2.7%	3	3
Bareilly	29	2.4%	8	14
Gaminara	25	2.1%	7	18
Typhimurium	19	1.6%	5	14
Muenster	16	1.3%	5	6
Cubana	10	0.8%	3	6

A lot of PFGE patterns!

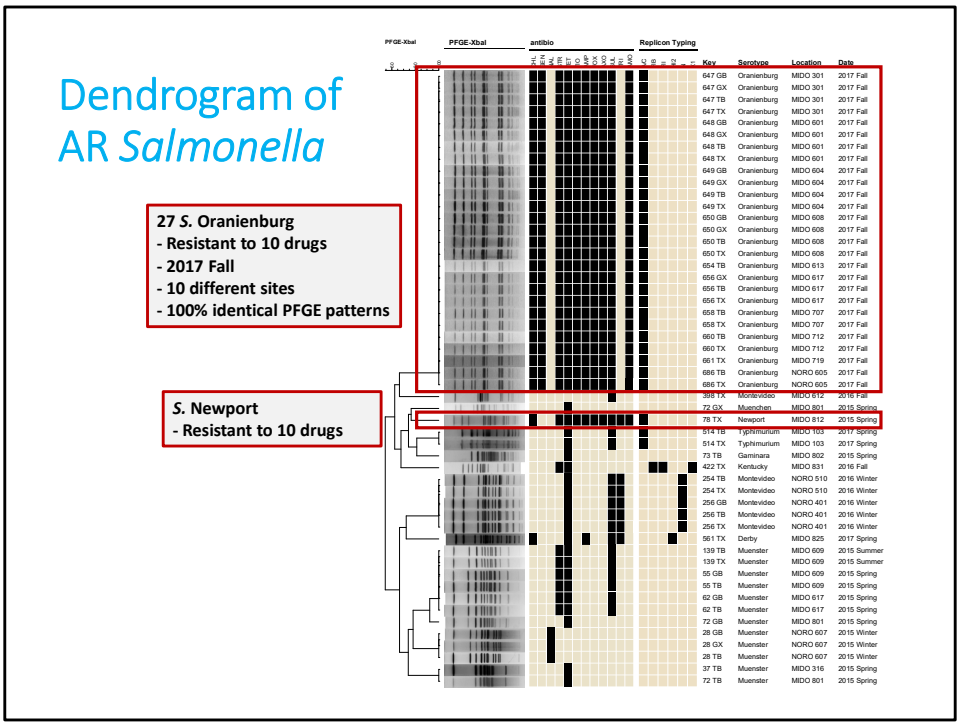
AR *Salmonella*

AR *Salmonella* (n= 52) recovery rate 4.4%

AR profiles[a]	No. of resistances	No. of isolates	Serotypes
Pan-susceptible	0	1138	
Nal	1	3	Muenster [3]
Sul	1	1	Montevideo [1]
Tet	1	5	Muenster [3], Muenchen [1], Gaminara [1]
StrTet	2	1	Kentucky [1]
SulTet	2	2	**27 *S.* Oranienburg - 2017 Fall - 10 different sites - 100% identical PFGE patterns**
StrSulTet	3	6	
SulTetTri	3	5	
AmpChlSulTetTri	5	1	
AmoAmpFoxTioAxoChlGenStrSulTet	10	27	Oranienburg [27]
AmoAmpFoxTioAxoChlStrSulTetTri	10	1	Newport [1]

[a] amoxicillin/clavulanic acid (Amo), ampicillin (Amp), cefoxitin (Fox), ceftiofur (Tio), ceftriaxone (Axo), chloramphenicol (Chl), gentamicin (Gen), nalidixic acid (Nal), streptomycin (Str), sulfisoxazole (Sul), tetracycline (Tet), trimethoprim/sulfamethoxazole (Tri)

This is the result of the susceptibility testing of Salmonella isolates. The recovery rate of AR Salmonella was 4.4% with 52 AR isolates. We have 1 S. Newport, which is resistant to 10 different drugs, and 27 S. Oranienburg, which are also resistant to 10 drugs. Interestingly, these S. Oranienburg isolates were all isolated from the 2017 Fall collection from 10 different sites, but they all seem to be clones with the 100% identical PFGE patterns.



The 52 Salmonella isolates were selected for further testing and this is the dendrogram of the R Salmonella with PFGE patterns, AR phenotypes, replicon types, and serotypes. We can see some clones, including 27 S. Oranienburg that were all isolated in the same season but from different sites. This is the MDR S. Newport with resistance to 10 different drugs and it has an A/C plasmid which is a large plasmid usually associated with MDR. MDR Salmonella Newport with A/C plasmid has caused several outbreaks in humans and cattle, so these bacteria were expected to be isolated from humans and animals, but not from surface water, but in fact, this isolate had a matching PFGE pattern as an outbreak strain.

Location of AR genes on MGEs

Isolate (serotype)	Gene[a]	location	Isolate (serotype)	Gene[a]	location
78 TX	(AGly) *aadA2*	integron (A/C)	561 TX	(AGly) *aadA2*	HI2
(Newport)	(Bla) bla_{CMY-2}	A/C	(Derby)	(Bla) bla_{TEM-1}	HI2
	(Tmt) *dfrA12*	integron (A/C)		(Tmt) *dfrA12*	HI2
	(Phe) *floR*	A/C		(Phe) *floR*	HI2
	(AGly) *strA*	A/C		(Sul) *sul1*	HI2
	(AGly) *strB*	A/C		(Tet) *tetB*	HI2
	(Sul) *sul1*	integron (A/C)	647 GB	(AGly) *aadB*	A/C
	(Sul) *sul2*	A/C	(Oranienberg)	(AGly) *aph3-Ia*	A/C
	(Tet) *tetA*	A/C		(Bla) bla_{CMY-2}	A/C
	(Tet) *tetR*	A/C		(Bla) bla_{TEM-1}	A/C
256 GB	(Tmt) *dfrA15*	N		(Phe) *cmlA5*	A/C
(Montevideo)	(Sul) *sul1*	N		(AGly) *strA*	A/C
	(Tet) *tetA*	N		(AGly) *strB*	A/C
	(Tet) *tetR*	N		(Sul) *sul2*	A/C
				(Tet) *tetA*	A/C
				(Tet) *tetR*	A/C

[a]class of antimicrobials: (Agly) aminoglycosides, (Bla) β-lactams, (Phe) phenicols, (Sul) sulfonamides, (Tet) tetracyclines, (Tmt) trimethoprim

4 isolates were selected for WGS and the locations of their resistance genes were identified. You can see that all of the isolates had their AR genes on specific plasmids while 3 AR genes of the MDR S. Newport isolate were located on an integron within the plasmid.

I was able to sequence only a set of 4 Salmonella isolates due to limited resources, but FDA is going to sequence all our 1,800 Salmonella isolates for the GenomeTrackr database which is like the CDC PulseNet database but instead of clinical isolates, it is a database of the WGS of the non-clinical isolates and managed by FDA. So we may have more interesting and comprehensive data in near future.

Serotype	total no. of isolates	no. of isolates with matching PFGE patterns (%)
Agona	1	1 (100%)
Anatum	36	8 (22.2%)
Aqua	7	0
Baildon	2	2 (100%)
Bareilly	30	18 (60%)
Berta	3	1 (33.3%)
Brandenburg	1	0
Braenderup	39	31 (79.5%)
Brazil	1	0
Cerro	2	2 (100%)
Cubana	14	1 (7.1%)
Derby	1	1 (100%)
Enteritidis	1	1 (100%)
Gaminara	25	0
Give	57	2 (3.5%)
Hartford	86	75 (87.2%)
Havana	3	3 (100%)
Infantis	22	15 (68.2%)
Inverness	5	2 (40%)
I 4,[5],12:b:-	53	40 (75.5%)
I 4,[5],12:i:-	3	3 (100%)
Javiana	3	3 (100%)
Kentucky	5	1 (20%)
Kiambu	3	0
Kintambo	1	1 (100%)
Liverpool	2	2 (100%)
Luciana	2	0
Mbandaka	9	9 (100%)
Meleagidis	2	2 (100%)
Mississippi	7	6 (85.7%)
Montevideo	113	90 (79.6%)
Muenchen	270	34 (12.6%)
Muenster	17	13 (76.5%)
Newport	99	77 (77.8%)
Oranienburg	32	32 (100%)
Orion	4	0
Ouakam	1	0
Paratyphi_B_var._L-tartrate+	1	0
Rough_O:i:-	1	0
Rubislaw	153	28 (18.3%)
Saintpaul	4	4 (100%)
Schwarzengrund	9	5 (55.6%)
Senftenberg	4	4 (100%)
Soerenga	5	5 (100%)
Tennessee	2	2 (100%)
Thompson	4	4 (100%)
Typhimurium	19	16 (84.2%)
Worthington	1	0
subspecies III (III_48:g,z51:-)	19	1 (5.3%)
subspecies IV	2	0

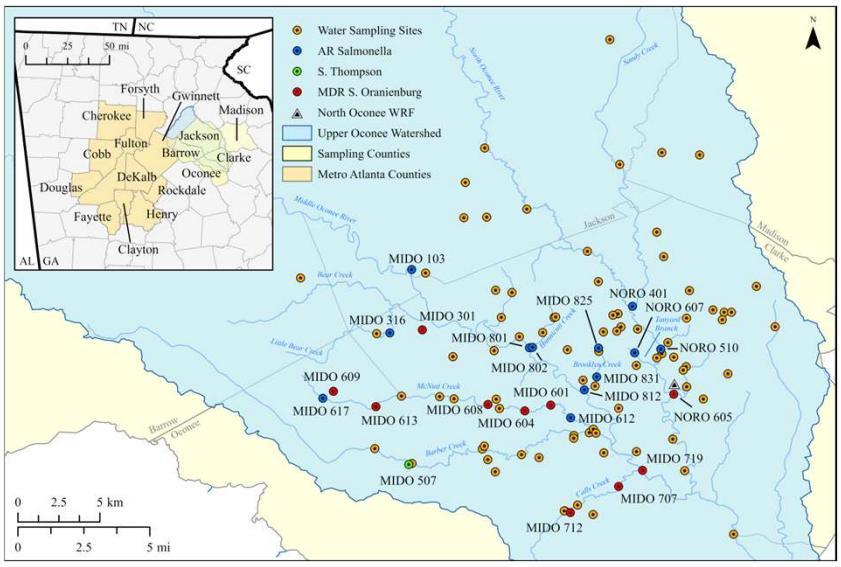
Table showing salmonella serotype (version 3) and PFGE patterns having indistinguishable PFGE patterns as clinical isolates.

About half (46.1%) of the isolates had PFGE patterns indistinguishable from human clinical isolates in the CDC PulseNet database.
There were several incidences where the same *Salmonella* strains with the same PFGE patterns were simultaneously recovered from both surface water and humans in the surrounding area (watershed and metro-Atlanta counties).

46.1% of *Salmonella* isolates have PFGE patterns that are indistinguishable from clinical isolates

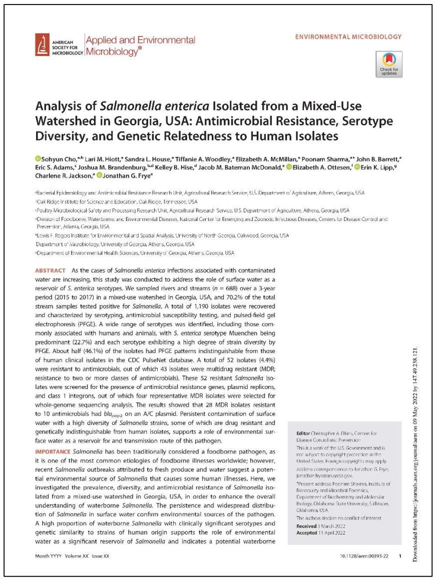
Several *Salmonella* strains with the same PFGE patterns were simultaneously recovered from surface water and humans in the surrounding area

Map of Sampling sites



On this map we can take a close look at those AR Salmonella as well as one S. Thompson I want to tell you about.
We got all of the MDR Oranienburg from a single sampling event; Shown in red, the majority of those came from sampling a single stream, McNutt's Creek, that is often known to be contaminated with Salmonella and high CFUs for E. coli.
There is a set of chicken houses upstream, but further upstream there are also cattle farms, additionally the same clone is detected at other unconnected streams, so no conclusions can be drawn.
As an example of Salmonella with matching patterns found in both our water samples and in humans at the same time, let's look at Salmonella Thompson, shown in green.
Its PFGE pattern was indistinguishable from one that caused an outbreak in Atlanta at the same time.
However, that outbreak was associated with a Greek restaurant.
It's possible a victim shed that bacterium into the water shed through leaky septic systems, however this is difficult to determine without epidemiological data that was not collected during the outbreak.

PFGE patterns of human isolates are indistinguishable from PFGE patterns of isolates from surface water



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ENVIRONMENTAL MICROBIOLOGY

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Analysis of *Salmonella enterica* Isolated from a Mixed-Use Watershed in Georgia, USA: Antimicrobial Resistance, Serotype Diversity, and Genetic Relatedness to Human Isolates

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ABSTRACT As the cases of *Salmonella enterica* infections associated with contaminated water are increasing, this study was conducted to address the role of surface water as a reservoir of *S. enterica* serotypes. We sampled rivers and streams (n = 688) over a 3-year period (2015 to 2017) in a mixed-use watershed in Georgia, USA, and 70.2% of the total stream samples tested positive for *Salmonella*. A total of 1,190 isolates were recovered and characterized by serotyping, antimicrobial susceptibility testing, and pulsed-field gel electrophoresis (PFGE). A wide range of serotypes was identified, including those commonly associated with humans and animals, with *S. enterica* serotype Muenchen being predominant (22.7%) and each serotype exhibiting a high degree of strain diversity by PFGE. About half (46.1%) of the isolates had PFGE patterns indistinguishable from those of human clinical isolates in the CDC PulseNet database. A total of 52 isolates (4.4%) were resistant to antimicrobials, out of which 43 isolates were multidrug resistant (MDR; resistance to two or more classes of antimicrobials). These 52 resistant *Salmonella* isolates were screened for the presence of antimicrobial resistance genes, plasmid replicons, and class 1 integrons, out of which four representative MDR isolates were selected for whole-genome sequencing analysis. The results showed that 28 MDR isolates resistant to 10 antimicrobials had $bla_{cmy\text{-}2}$ on an A/C plasmid. Persistent contamination of surface water with a high diversity of *Salmonella* strains, some of which are drug resistant and genetically indistinguishable from human isolates, supports a role of environmental surface water as a reservoir for and transmission route of this pathogen.

IMPORTANCE *Salmonella* has been traditionally considered a foodborne pathogen, as it is one of the most common etiologies of foodborne illnesses worldwide; however, recent *Salmonella* outbreaks attributed to fresh produce and water suggest a potential environmental source of *Salmonella* that causes some human illnesses. Here, we investigated the prevalence, diversity, and antimicrobial resistance of *Salmonella* isolated from a mixed-use watershed in Georgia, USA, in order to enhance the overall understanding of waterborne *Salmonella*. The persistence and widespread distribution of *Salmonella* in surface water confirm environmental sources of the pathogen. A high proportion of waterborne *Salmonella* with clinically significant serotypes and genetic similarity to strains of human origin supports the role of environmental water as a significant reservoir of *Salmonella* and indicates a potential waterborne

Editor Christopher A. Elkins, Centers for Disease Control and Prevention

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The authors declare no conflict of interest.
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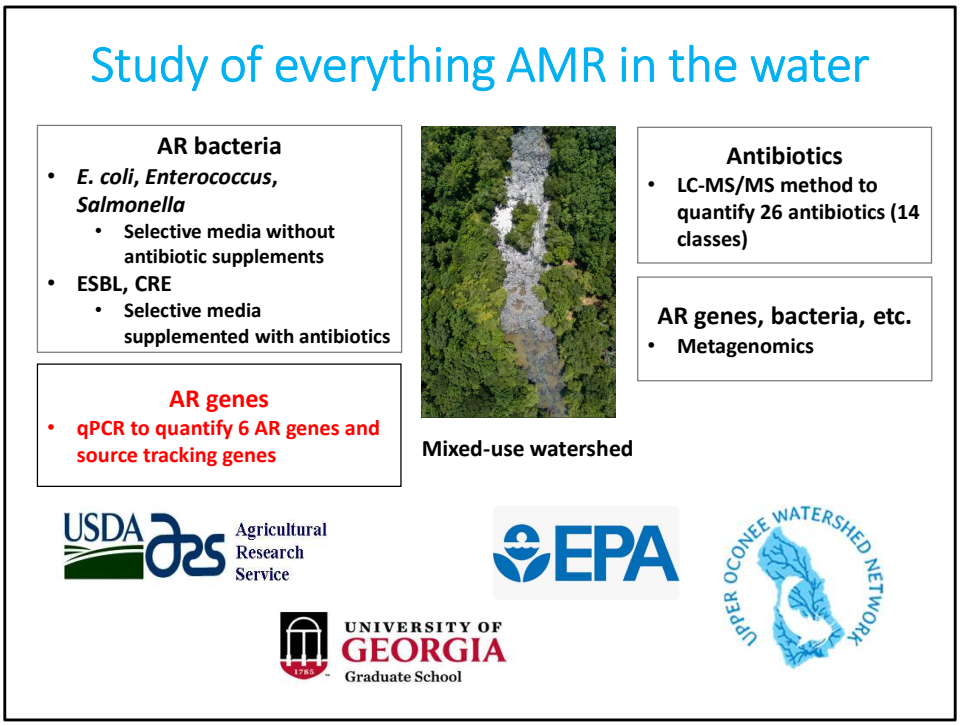


We not only isolated ARB from surface water to characterize the bacteria that are culturable and express their ARGs, but also quantified the total ARGs present within the whole bacterial populations in the watershed, including those that have not been expressed.

In addition, antibiotics that are important to human and veterinary medicine were measured to investigate the occurrence and distribution of these antibiotics in aquatic environments.

As WWTPs have been proposed to be the hotspots for the emergence of ARB and from where these bacteria are spread into the natural environment, influents and effluents from three WWTPs located within the watershed and whose effluents flow into the streams within the watershed were included in the analyses.

Water samples positive for AR genes

AR genes	No. of water samples positive for AR genes (%)			
	Fall (n = 38)	Winter (n = 38)[a]	Spring (n = 34)[a]	Summer (n = 40)
ermB	23 (60.5)	8 (21.6)	8 (23.5)	11 (27.5)
tetB	10 (26.3)	2 (5.6)	1 (2.9)	2 (5.0)
bla_{KPC}	9 (23.7)	5 (13.5)	2 (5.9)	7 (17.5)
bla_{SHV}	9 (23.7)	2 (5.4)	1 (2.9)	2 (5.0)
qnrS	8 (21.1)	8 (21.1)	3 (9.4)	8 (20.0)
bla_{CTX-M}	3 (7.9)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Some samples could not be analyzed for certain AR genes due to technical issues

The most frequently detected resistance gene was *ermB*, which was present in 33.3% (50/150) of the total water samples tested. This was followed by *qnrS* (18.2%; 27/148), bla_{KPC} (15.4%; 23/149), *tetB* (10.1%; 15/148), bla_{SHV} (9.4%; 14/149) and bla_{CTX-M} (2.0%; 3/148).

WWTP samples are not presented because of low no. of samples tested: 9 influent and 6 effluent samples. All influent samples were positive for every AR gene, while a few effluent samples were negative for blaCTX-M and tetB genes.

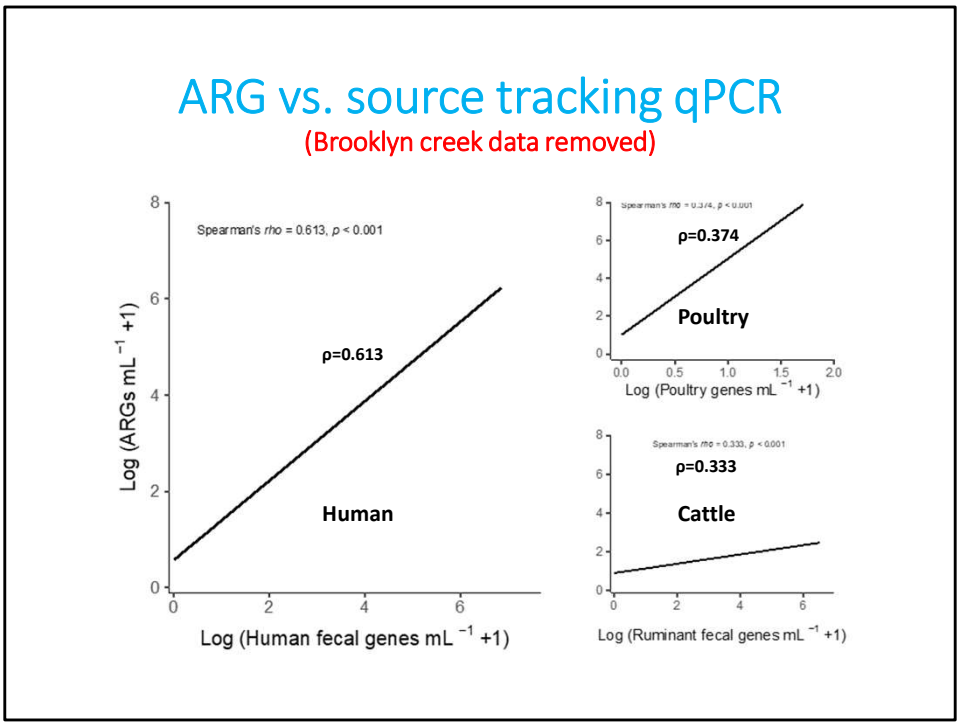
AR gene copy numbers in water

AR genes	gene copy numbers in water samples (copies/mL)							
	Fall (n = 38)		Winter (n = 38)		Spring (n = 34)		Summer (n = 40)	
	maximum	average	maximum	average	maximum	average	maximum	average
ermB	1,533.8	57.2	355.0	11.3	41.0	1.9	347.5	11.9
tetB	127.0	4.0	0.7	0.0	1.6	0.0	1.0	0.1
bla_{KPC}	13.5	1.6	49.9	2.0	76.2	2.4	377.5	9.8
bla_{SHV}	325.0	10.7	2.0	0.1	2.0	0.1	7.7	0.2
qnrS	308.4	12.5	582.6	19.5	122.4	4.6	703.0	21.5
bla_{CTX-M}	455.2	15.9	0.0	0.0	0.0	0.0	0.0	0.0

While ARG copy numbers ranged from 10^0 to 10^3 copies/mL in surface water, the copy numbers ranged from 10^3 to 10^5 copies/mL in influents and 10^0 to 10^5 copies/mL in effluents.

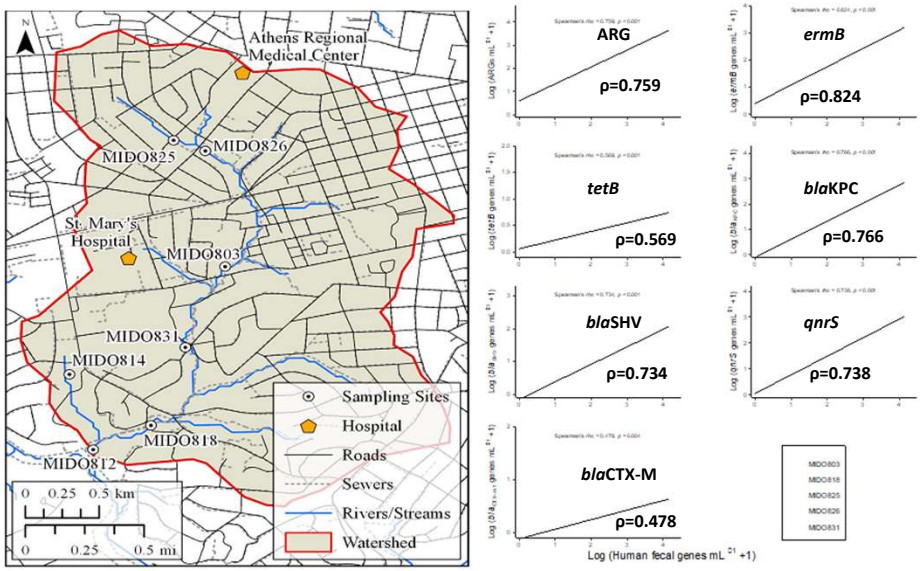
qPCR data on ARGs and source tracking genes

- **Do ARGs in surface water correlate with source poultry, cattle, or human source tracking genes?**
- **Do ARGs contaminating surface water correlate with land use?**
- **Do ARGs and source tracking genes contaminating surface water correlate with the sanitary sewer or septic systems?**



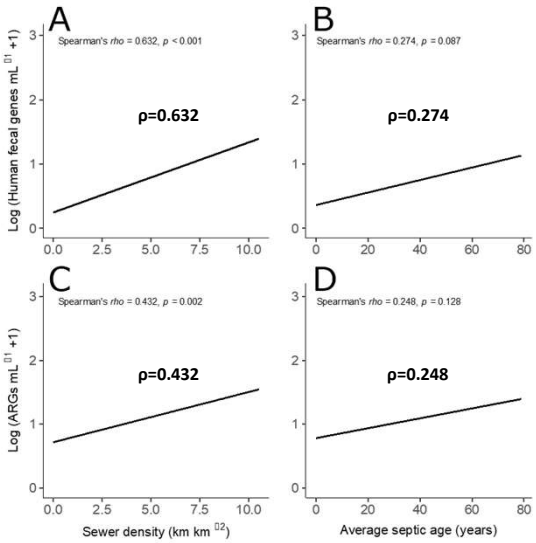
This is a comparison of AR genes qPCR with source tracking qPCR for the whole watershed except for the Brooklyn creek samples.
You can see that human fecal genes correlate with AR genes strongly with a rho of .613.
Poultry and cattle fecal genes weakly correlate with AR genes.
Therefore, AR genes are associated with human feces more than poultry or cattle.

Brooklyn Creek qPCR



We removed Brooklyn Creck from the data on the previous slide, because we were afraid it would skew the results for the whole watershed.
Brooklyn Creek is in a residential area, and you will recognize it and remember it's the same area we isolated that ST131 with the ESBL, because you can seen that Athens Regional is up here, and Saint Mary's hospital is in the middle.
Here the association between AR genes and human fecal markers is very high with a rho of 0.759.
In fact the association is so strong we can look at specific genes, with ermB having a rho of 0.824.
Others include tetB, KPC, SHV qnrS, and CTX-M.
So it appears that human waste is strongly associated with AR genes in the watershed, especially in these residential areas.

Human and ARG qPCR vs. sewer density and septic age



We tried to correlate the data with land use, and really got nothing significant.
However, if we compared human fecal genes with sewer density we got a strong association with a rho of 0.632.
If we compare AR genes to sewer density we also get an association but it is weaker.
When we compare these markers to average septic age, we get a very weak association.
Therefore, much of the surface water contamination with human markers and AR genes is likely due to our aging and leaking sewer system.
And this is a problem for most cities in America due to the lack of maintenance of our public infrastructure.

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Non-point source fecal contamination from aging wastewater infrastructure is a primary driver of antibiotic resistance in surface waters

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ABSTRACT

Antibiotic resistance is a global threat to human health. Many surface water resources are environmental hotspots of antibiotic resistant gene (ARG) transfer, with agricultural runoff and human waste highlighted as common sources of ARGs to aquatic systems. Here we quantified fecal marker genes and ARGs in 992 stream water samples collected seasonally during a 5-year period from 115 sites across the Upper Oconee watershed (Georgia, USA), an area characterized by gradients of agricultural and urban development. Widespread fecal contamination was found from humans (46% of samples), ruminants (55%), and poultry (19%), and 71% of samples tested positive for at least one of the six targeted ARGs (*ermB*, *mefA*, *blaCTX-M-1*, *blaTEM*, *blaSHV*, and *gyrA*). While ARGs were strongly correlated with human fecal markers, many highly contaminated samples were not associated with sewage outfalls, an expected source of fecal and ARG pollution. To determine sources of contamination, we synthesized ARG and fecal marker data with geospatial data on land use/land cover and wastewater infrastructure across the watershed. This novel analysis found strong correlations between ARGs and measures of sewer density, sewer length, and septic system age within sample watersheds, indicating non-point sources of fecal contamination from aging wastewater infrastructure can be critical disseminators of anthropogenic ARGs in the environment.

1. Introduction

The widespread use and over-use of antibiotics in human health and agriculture has made antimicrobial resistance (AMR) an ongoing global crisis (Antimicrobial Resistance Collaborators, 2022; O'Neill, 2016; World Health Organization, 2021). Resistance has been reported for nearly all known antibiotics (Ventola, 2015), and annual deaths from resistant infections are projected to reach 10 million by 2050 (O'Neill, 2016). To address AMR and develop mitigation strategies, it is critical to understand reservoirs and dissemination pathways of AMR. A growing body of research identifies the environment as both a recipient and source of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). In particular, surface waters such as streams and rivers are an ideal setting for AMR dissemination, as they are not only impacted by anthropogenic activities but are also dynamic environments with a high potential for exchange of mobile genetic elements and

Abbreviations: ARB, antibiotic resistant bacteria; ARG, antibiotic resistance gene; AMR, antimicrobial resistance; PCA, principal component analysis; qPCR, quantitative PCR; UOWN, Upper Oconee Watershed Network; WWTP, wastewater treatment plant.

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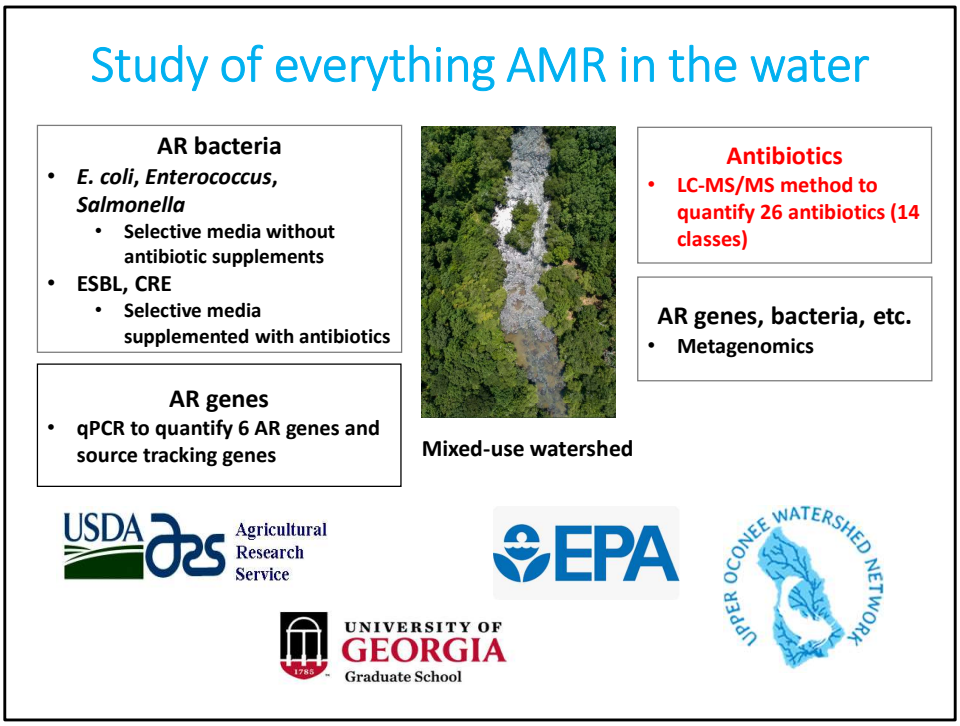
Non-point source fecal contamination from aging wastewater infrastructure is a primary driver of antibiotic resistance in surface waters

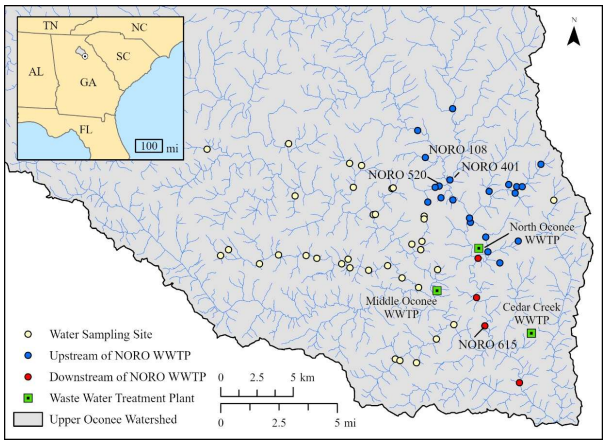


We not only isolated ARB from surface water to characterize the bacteria that are culturable and express their ARGs, but also quantified the total ARGs present within the whole bacterial populations in the watershed, including those that have not been expressed.

In addition, antibiotics that are important to human and veterinary medicine were measured to investigate the occurrence and distribution of these antibiotics in aquatic environments.

As WWTPs have been proposed to be the hotspots for the emergence of ARB and from where these bacteria are spread into the natural environment, influents and effluents from three WWTPs located within the watershed and whose effluents flow into the streams within the watershed were included in the analyses.

Map of water sampling sites and WWTPs



Cho et al. 2023. Antibiotics.

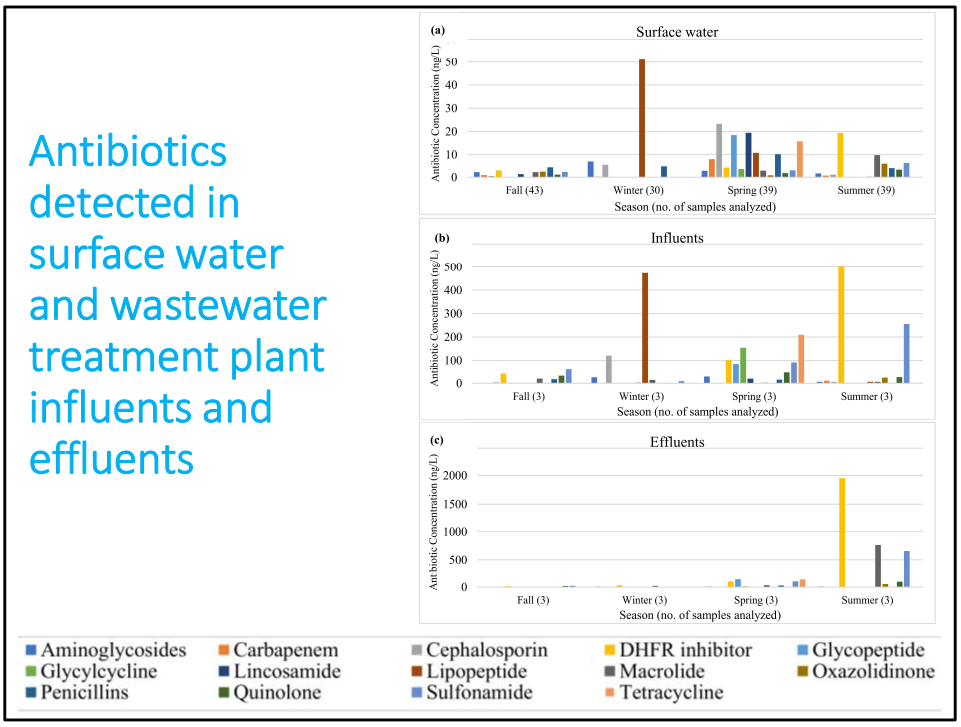
Wastewater samples from 3 wastewater treatment plants (WWTPs) within the watershed

In order to find the source of the AR contaminants in the surface water, we chose WWTPs. We collected influent and effluent samples from 3 WWTPs located within the watershed and whose effluents flow into the streams within the watershed. And we investigated whether WWTPs were effective in reducing AR contaminants and whether WWTPs contributed to the levels of AR contaminants in surface water.

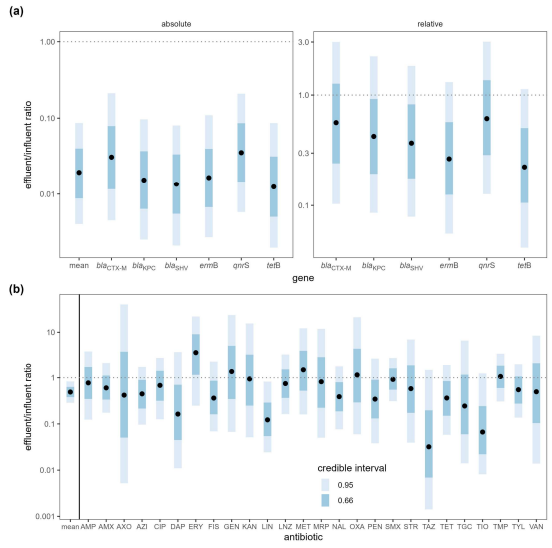
Antibiotic detection in water samples

- **All 26 antibiotics tested were detected in at least one sample**
- **At least one antibiotic was detected in each sample tested**
- **Spikes in human associated antibiotics were seen in the Spring**
- **A spike in sulfamethoxazole and trimethoprim was detected in the Summer**

Antibiotics detected in surface water and wastewater treatment plant influents and effluents



AMR gene copy numbers detected (normalized to 16SrDNA)



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If you look at the upper figures to look at AR gene copy number ratio between wastewater influent and effluent samples, wastewater treatment greatly reduced the absolute copy numbers of ARGs but did not significantly change their relative copy numbers, which was normalized to 16S rRNA gene copy numbers. The absolute abundance of ARGs was potentially decreased due to a reduction in the overall abundance of bacterial populations during the treatment process. However, high density of bacteria and nutrients as well as antibiotics within the treatment system could have led to a favorable environment for horizontal gene transfer of ARGs and therefore a smaller reduction in the relative abundance of ARGs.

But when the antibiotic concentrations in the influent and effluent samples were compared as shown in the bottom figure, WWTPs did not remove antibiotics as efficiently as ARGs. The average antibiotic concentration decreased by half, but this difference was driven by small decreases in most of the antibiotics, although most of these decreases were not statistically significant on an individual basis except for lincomycin.

WWTPs as a source of AR contaminants?

- **When WWTP influent samples and effluent samples were compared:**
  - **WWTPs only partially removed AR bacteria, AR genes, and antibiotics, with the effluents containing high levels of AR contaminants**
  - **WWTPs were not very effective in removing AR contaminants, releasing the contaminants into receiving water**
- **When water samples collected upstream and downstream of WWTP were compared:**
  - **No significant differences in AR contaminants between the upstream samples and downstream samples**

But when we compared the water samples collected upstream and downstream of a WWTP, there was no significant difference in AR contaminants between the upstream samples and downstream samples.
Also, only a small number of the water sampling sites received wastewater effluents, but most of the sites contained high levels of AR contaminants throughout the year, indicating that there are other sources of AR pollution apart from WWTPs.

So our conclusion was that although WWTPs contribute to the AR contamination in surface water, they are not the main source of AR in surface water of the Upper Oconee Watershed.

Published in Antibiotics

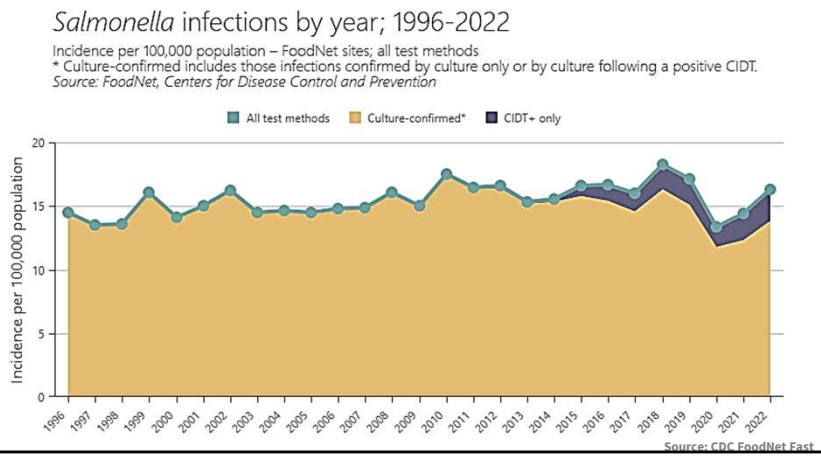
This paper on the presence and distribution of AR bacteria, AR genes, and antibiotics in surface water and the impact of WWTPs on AR in surface water has been just accepted for publication. So, if you are interested, you could learn more about this in this paper: Distribution of Antibiotic Resistance in a Mixed-Use Watershed and the Impact of Wastewater Treatment Plants on Antibiotic Resistance in Surface Water.

How is this surveillance data used in One Health approach?

- **The National Antimicrobial Resistance Monitoring System has no means to integrate this data**
  - **Where did the *Salmonella* in the water come from?**
  - **Agriculture is the assumed source, but sewers leak and wild animals poop, both can be a source of *Salmonella***
  - **What is the risk that *Salmonella* found in surface water will contaminate food and infect a human?**
- **Current efforts to reduce *Salmonella* infections**
  - **USDA Food Safety Inspection Service: eliminate *Salmonella* from U.S. poultry trough regulations**

Salmonellosis has not decreased

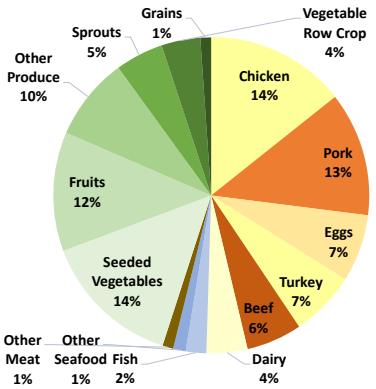
- ***Salmonella* causes about 1.3 Million infections each year**
- **Healthy People 2010 goal reduced *Salmonella* positive carcasses by 50%**
- **Numbers of human infections were unchanged**
- **Healthy People 2030 goal: reduce human cases by 25%**



Salmonella causes about 1.3 Million infections each year, most of which are caused by specific serotypes.
The Healthy People 2030 goal is to reduce human infections by 25%, while poultry only causes ~21% of cases as seen on the previous slides.
We have been trying to reduce Salmonella infections for the past three decades, and the goals of reduction of Salmonella in retail poultry products have been successful, but the levels of Salmonella infections in humans have not decreased.

Sources of the *Salmonella*?

- Highly diverse pathogen; ~2,600 different serotypes
- Found in many hosts and environments
- Rarely causes disease in host animals
- *Salmonella* source attributions (estimated):
  - Green: Produce, Fruits, & Vegetable sources
  - Blue: Fish & Seafood
  - Cream: Dairy
  - Brown: Beef
  - Orange: Pork
  - Eggs: Tan
  - Light Yellow: Poultry (14% Chicken & 7% Turkey)

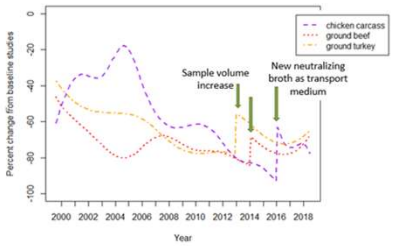


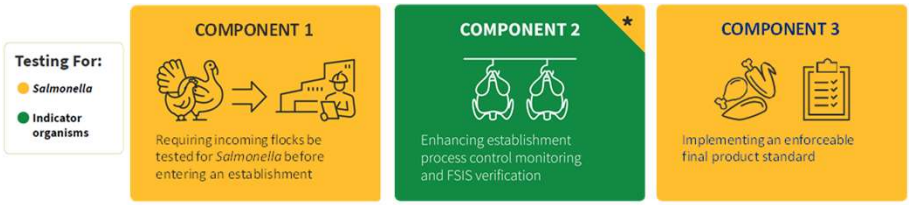
Estimated *Salmonella* source attribution
FDA 2020

Salmonella is a gram-negative, rod-shaped bacteria, that is highly diverse and includes over 2,600 serotypes.
According to FDA, Salmonella infections that could be associated with food fell into these percentages.
Meat, fish, seafood, and other meat accounted for roughly 54% of attributed human infections in the U.S.
About 21% of human infections were associated with poultry.
Turkeys, shown in yellow, account for 7% and chickens, also shown in yellow accounting for 14% of Salmonella infections.
It is interesting to note that almost half of Salmonella infections are associated with products other than meat.
And we need to keep in mind that attribution is difficult and usually relies on a large enough outbreak to trigger an investigation, while most Salmonella infections are sporadic and never linked to a source.
Therefore, these numbers may not represent the true effect of the different commodities on human Salmonella infections.

Strategies to reduce human infections

- **Despite the downward trend in *Salmonella* contamination on meat**
- **Disconnect between contamination and illness**
- **2021 FSIS proposed a new framework**

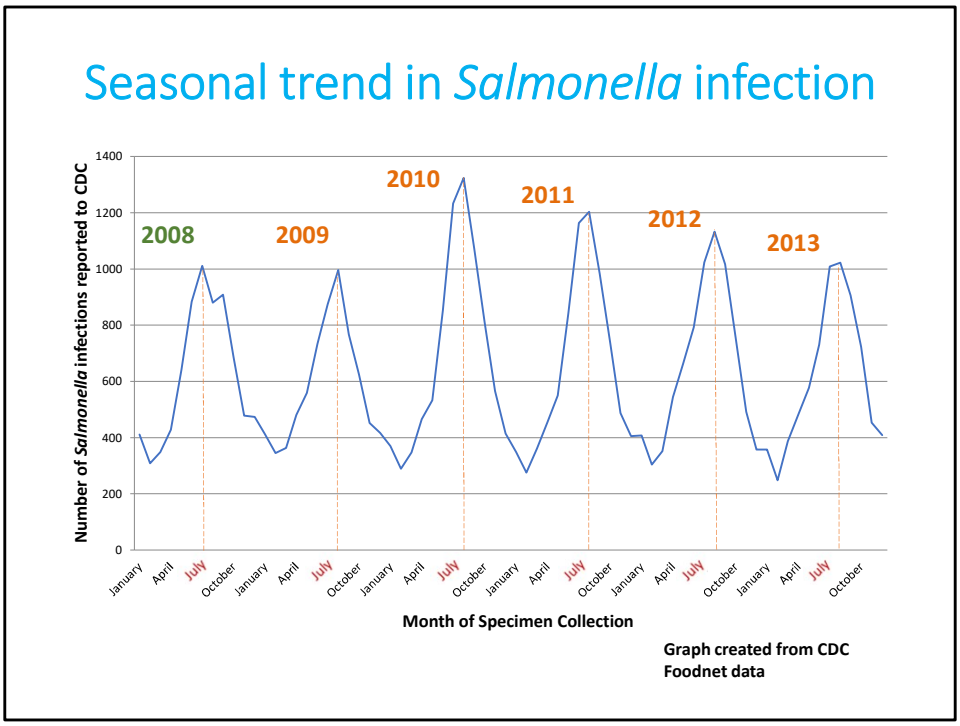




*Under this proposed framework, testing for *Salmonella* would also occur during the same steps in production as testing for indicator organisms.

Source: FSIS Bill Shaw

- The persistence of salmonella illness is occurring despite less contamination of meat
- The posted framework under consideration, uses three strategies to target *Salmonella* at different points in the slaughter and processing operation.
  - Testing for *Salmonella* before entering an establishment.
  - Enhancing establishment process control monitoring and FSIS verification.
  - Implementing an enforceable final product standard that includes serotype and quantification.
  - So our conversation today about what interventions work best and what indictors are most useful is very timely.



This graph shows a seasonal trend in Salmonella infections.
Y-axis represents the number of Salmonella infections reported to CDC and X-axis represents the month the specimens were collected.
There is a clear pattern repeating every year with more infections during summer months.
Why would a food borne disease be seasonal?
There simply can't be that much potato salad, plus, most cases reported are not associated with an outbreak and are what we call sporadic cases with no known source.
So what are some other reasons summer months it goes from less than 400 to nearly three times that?
Other things people do in the warm months is outdoor recreation, and a lot of that has contact with surface water, including swimming, fishing, boating, etc.
So, why not look for the cause of this increase in the environment?

46.1% of *Salmonella* isolates have PFGE patterns that are indistinguishable from clinical isolates

Several *Salmonella* strains with the same PFGE patterns were simultaneously recovered from surface water and humans in the surrounding area

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Any questions?