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Communication

Antibiotic-Resistant Enteric Bacteria in Environmental Waters

Lisa M. Casanova [1,*] and Mark D. Sobsey [2]

[1] Division of Environmental Health, School of Public Health, Georgia State University, P.O. Box 3984, Atlanta, GA 30303, USA

[2] Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina Chapel Hill, CB #7431, Chapel Hill, NC 27599, USA; sobsey@email.unc.edu

* Correspondence: lcasanova@gsu.edu; Tel.: +404-413-1136

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Abstract: Sources of antibiotic resistant organisms, including concentrated animal feeding operations (CAFOs), may lead to environmental surface and groundwater contamination with resistant enteric bacteria of public health concern. The objective of this research is to determine whether *Salmonella*, *Escherichia coli*, *Yersinia enterocolitica*, and enterococci resistant to clinically relevant antibiotics are present in surface and groundwater sources in two eastern North Carolina counties, Craven and Wayne. 100 surface and groundwater sites were sampled for *Salmonella*, *E. coli*, and enterococci, and the bacteria isolated from these samples were tested for susceptibility to clinically relevant antibiotics. *Salmonella* were detected at low levels in some surface but not groundwater. *E. coli* were in surface waters but not ground in both counties. Enterococci were present in surface water and a small number of groundwater sites. *Yersinia* was not found. Bacterial densities were similar in both counties. For *Salmonella* in surface water, the most frequent type of resistance was to sulfamethoxazole. There was no ciprofloxacin resistance. There were a few surface water *E. coli* isolates resistant to chloramphenicol, gentamicin, and ampicillin. Enterococci in surface water had very low levels of resistance to vancomycin, chloramphenicol, ampicillin, and streptomycin. *E. coli* and enterococci are present more frequently and at higher levels in surface water than *Salmonella*, but groundwater contamination with any of these organisms was rare, and low levels of resistance can be found sporadically. Resistant bacteria are relatively uncommon in these eastern N.C. surface and groundwaters, but they could pose a risk of human exposure via ingestion or primary contact recreation.

Keywords: *Salmonella*; CAFO; water; antibiotic resistance; surface water; groundwater

1. Introduction

Hog production is a major North Carolina industry, with 8.7 million hogs in the state as of 2016, second only to Iowa [1]. Over time, hog production has become more integrated and consolidated, and is now conducted mostly in large concentrated animal feeding operations (CAFOs) that can house thousands of hogs each [2]. Hog waste is managed by storing in large lagoons for several months [3], allowing anaerobic decomposition and microbial dieoff to take place before the waste is land applied on adjacent land [4]. Hog harbor and excrete fecally several kinds of bacteria that are potential human pathogens [5]. Lagoon wastewaters have been found to contain bacterial pathogens and antibiotic resistance genes [6,7]. Studies of North Carolina hog farms have found *Salmonella*, including antibiotic resistant *Salmonella*, in hogs, the farm environment, and waste lagoons [8]. Both lagoon leakage and land application of wastes may create opportunities for transport of bacteria to surface and groundwater sources [9,10], which may serve as a route of exposure for humans [11].

While enteric bacteria resistant to clinically relevant antibiotics have been identified in hog wastes and on farm water sources, it is less clear whether these bacteria are present in environmental surface and ground waters that are not on or adjacent to CAFOs. Identifying the presence and resistance patterns of enteric bacteria in non-CAFO environmental surface and ground waters is a first step to determining the scope of antibiotic resistance and its possible sources in environmental waters not associated with CAFOs. In North Carolina, hog production has historically been concentrated in the eastern part of the state [12]. Therefore, the objective of this research is to determine whether *Salmonella*, *Escherichia coli*, and enterococci resistant to clinically relevant antibiotics are present in non-CAFO surface and groundwater sources in two eastern North Carolina counties, Craven and Wayne, that have different densities of hog production operations.

2. Materials and Methods

2.1. Water Sampling

Samples were collected from July 2001 to February 2002. Sampling sites were surface and groundwater sources in Craven County and Wayne County that were not located on or adjacent to hog production sites. Ground water sites were private and monitoring wells; surface water sites were stream waters. Craven County is approximately 744 square miles and as of 2002 had approximately 20 hog operations with production totaling approximately 446,000 head. Wayne County is approximately 557 square miles and as of 2002 had approximately 92 hog production operations consisting of approximately 1.4 million head [13]. From July to November 2001, a total of 50 sites were sampled in Craven County, consisting of 16 surface water sites and 34 ground water sites. From August 2001 to February 2002, 50 sites were sampled in Wayne County, consisting of 21 surface water sites and 29 ground water sites. Each site was sampled once, and one grab sample was taken from each site on the sampling day. Water was collected in sterile containers, placed on ice immediately, and shipped overnight to the laboratory, where it was processed within 8 h of arrival.

2.2. Isolation of Salmonella

Samples of water were analyzed for *Salmonella* using the most probable number (MPN) method [14]. Aliquots of 200, 20, and 2 mL of sample were placed in equal volumes of buffered peptone water and incubated at 37 °C for 24 h. Sample volumes of 100 µL were then transferred from each bottle of buffered peptone water to tubes containing 10 mL of Rappaport-Vasiliadis selective enrichment broth (Becton Dickinson Co., Sparks, MD, USA) and incubated at 41 °C for 24 h [15]. Each tube of RV broth was then streaked onto *Salmonella*-Shigella (SS) agar (Becton Dickinson) and incubated at 41 °C for 24 h. If available, four colonies of presumptive *Salmonella* from each sample were purified by sequential streaking on TSA and frozen at −80 °C in glycerol solution for antimicrobial susceptibility testing. Isolates were confirmed biochemically to be *Salmonella* using the Enterotube II system. Isolates positively identified as *Salmonella* were then tested for antimicrobial susceptibility, and density of *Salmonella* in the sample was calculated using the Thomas equation [16].

2.3. Isolation of E. coli

Samples were analyzed for *E. coli* using the membrane filtration method. Five hundred milliliter aliquots of groundwater or 100 mL and 10 mL aliquots of surface water were filtered through 0.45 µm pore size filters and placed on mFC agar (Becton Dickinson Co.). Plates were incubated at 37 °C for 2 h to resuscitate stressed or injured organisms, then incubated at 44 °C for an additional 22 h. Blue colonies were counted, and the filters were then transferred onto nutrient agar plates supplemented with MUG (Becton Dickinson Co.). Plates were incubated at 37 °C for 4 h. Plates were then examined under ultraviolet light, and blue colonies that fluoresced were counted as presumptive *E. coli*. If available, four colonies of presumptive *E. coli* from each sample were purified by sequential streaking on TSA and frozen at −80 °C in glycerol solution for antimicrobial susceptibility testing. Isolates were confirmed to

be *E. coli* using the Enterotube II system for the identification of *Enterobacteriaceae* (Becton-Dickinson). Isolates positively identified as *E. coli* were then tested for antimicrobial susceptibility.

2.4. Isolation of Yersinia enterocolitica

Samples were analyzed for *Yersinia enterocolitica* using an enrichment procedure [17]. Aliquots of 300, 30, and 3 mL of sample were placed in equal volumes of pre-enrichment medium (PEM) (For 1 L, 20 g yeast extract, 10 g Oxoid special peptone, 7.1 g Na_2HPO_4, 1 g NaCl, 1g KCl, 10 mL of 0.1% *w*/*v* $MgSO_4 \cdot 7H_2O$, and 10 mL of 0.1% *w*/*v* $CaCl_2 \cdot 2H_2O$. Add dry ingredients to 980 mL water, boil to dissolve, and bring to pH 8.0. Autoclave, cool to 50 °C, and aseptically add $MgSO_4$ and $CaCl_2$ solutions).

The PEM was incubated at 4 °C for 7 days. 100 mL of sample were then transferred from each bottle PEM to tubes containing 100 mL of *Yersinia* selective broth (YSB) (For 1 L, 37 g brain heart infusion broth, 10 mL of 0.1% *w*/*v* $MgSO_4 \cdot 7H_2O$, 10 mL of 0.1% *w*/*v* $CaCl_2 \cdot 2H_2O$, and 0.8 g potassium chlorate. Prepared the same as PEM). The YSB was incubated at 4 °C for 7 days. Each bottle of YSB was then streaked onto Celsulfodin Irgasan Novobiocin (CIN) agar (Becton Dickinson) and incubated at 28 °C for 48 h. Colonies having the typical appearance of *Yersinia* were streaked onto MacConkey agar (Becton Dickinson) and incubated at 28 °C for 48 h. Non-lactose-fermenting colonies were streaked onto TSA and grown at 37 °C for 24 h.

Oxidase tests (Becton Dickinson) were conducted on colonies from TSA. Oxidase negative colonies were inoculated into Kliger iron agar (KIA), Simmons citrate agar, Christensen's urea agar, phenylalanine deaminase agar, and motility-indole-ornithine agar (all from Becton Dickinson). If available, four colonies of presumptive *Y. enterocolitica* from each sample were purified by sequential streaking on TSA and frozen at −80 °C in glycerol solution for antimicrobial susceptibility testing. Isolates were confirmed to be *Y. enterocolitica* using the API 20E system for the identification of *Enterobacteriaceae* (Bio-Merieux). Isolates identified as *Yersinia* species using the API 20E were confirmed as *Y. enterocolitica* by their fermentation pattern using celbiose, melibiose, rhamnose, and sucrose to screen out environmental *Yersinia* species.

2.5. Antimicrobial Susceptibility Testing

Four isolates from each water sample were tested for antimicrobial susceptibility. If fewer than four colonies of any organism were able to be isolates from a sample, all available isolates were tested. Antimicrobial susceptibility testing was done by the broth microdilution method [18] using a Sensititre custom antimicrobial susceptibility testing plate (Trek Diagnostic Systems, Westlake, OH, USA) according to the manufacturer's instructions. Antibiotics tested were chosen for clinical relevance. *Salmonella* and *E. coli* were tested against trimethoprim (TMP), sulfamethoxazole (SMX), chloramphenicol (CHL), gentamicin (GEN), ampicillin (AMP), ciprofloxacin (CIP), and ceftriaxone (CEF). Enterococci were tested against vancomyin (VAN), streptomycin (STR), chloramphenicol (CHL), gentamicin (GEN), and ampicillin (AMP). Minimum inhibitory concentrations (MIC) were recorded, and susceptibility or resistance was determined using breakpoints established by the Clinical and Laboratory Standards Institute [19]. As recommended by the susceptibility testing system manufacturer, American Type Culture Collection strains *E. coli* 25922, *Pseudomonas aeruginosa* 27853, *Enterococcus* faecalis 29212, and *Staphylococcus aureus* 29213 (American Type Culture Collection, Rockville, MD, USA) were used for quality control.

3. Results

Surface and Ground Waters

Bacterial densities in water are shown in Table 1. Mean values are for sites that had detectable bacteria (0 values are excluded). *Salmonella* was not detected in any groundwater samples; some surface water samples were positive for *Salmonella* at low levels. *E. coli* were isolated in surface waters

in both counties, but not groundwater. Enterococci was found in surface water in both counties and a small number of groundwater sites in Wayne. *Yersinia* was not isolated from any sample sites.

Table 1. Bacteria in surface and groundwater sites.

County	Source	Total Sites	*Salmonella*		*E. coli*		*Enterococcus*	
			Present	Mean (Range) (log_{10} MPN/100 mL)	Present	Mean (Range) (log_{10} CFU/100 mL)	Present	Mean (Range) (log_{10} CFU/100 mL)
Craven	surface	16	10	−0.31 (−1.00–0.75)	15	1.95 (0.54–2.72)	15	2.08 (1.17–2.86)
	ground	34	0	-	0	-	0	-
Wayne	surface	20	5	−0.78 (−0.16–1.00)	20	1.70 (0.86–3.00)	14	1.71 (1.24–2.63)
	ground	30	0	-	0	-	3	1.05 (0.41–2.05)

From the 15 surface water sites where *Salmonella* was detected, 43 isolates were collected and tested for antimicrobial susceptibility (Table 2). The most frequent type of resistance was to sulfamethoxazole, found in isolates from Craven County sites. No resistance to ciprofloxacin was detected. Only one isolate was resistant to two antibiotics (sulfamethoxazole and ampicillin). Due to a shortage of testing supplies from the manufacturer, *Salmonella* was not tested against ceftriaxone.

Table 2. Antimicrobial resistance in *Salmonella* isolates from surface water.

Source	Total Isolates	TMP		Resistant Isolates						GEN		AMP	
				SMX		CHL		CIP					
		n	%	n	%	n	%	n	%	n	%	n	%
Craven	32	0	0	9	28	0	0	0	0	0	0	1	3
Wayne	11	0	0	0	0	0	0	0	0	0	0	0	0

From the 35 surface water sites positive for *E. coli*, 92 isolates were tested for antibiotic resistance (Table 3). There were a few isolates resistant to chloramphenicol, gentamicin, and ampicillin; no isolates were resistant to more than one antibiotic. No resistance to ciprofloxacin or ceftriaxone was detected.

Table 3. Antimicrobial resistance in *E. coli* isolates from surface water.

Source	Total Isolates	TMP		Resistant Isolates						GEN		AMP	
				SMX		CHL		CIP					
		n	%	n	%	n	%	n	%	n	%	n	%
Craven	42	0	0	0	0	2	4	0	0	0	0	2	4
Wayne	50	0	0	0	0	2	4	0	0	1	0	1	2

From the 29 surface water and 3 groundwater sites where enterococci were detected, 120 isolates were tested for resistance (Table 4). Very low levels of resistance to vancomycin, chloramphenicol, ampicillin, and streptomycin were found; no isolates were resistant to more than one antibiotic. These resistant isolates were all from surface water. Vancomycin resistant enterococci (VRE) are of high public health importance; however, identification of acquired vs. intrinsic vancomycin resistance is highly dependent on accurate speciation. Most isolates in this study were identified using biochemical methods as *E. faecalis* or *E. faecium*, with a few isolates of *E. gallinarum*. *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin. Further species confirmation may be needed before definitive designation of these isolates as VRE.

Overall, bacterial densities were similar in Craven and Wayne county waters. Although enterococci were found at low levels in Wayne County groundwater only, the number of positive samples was very small, making it difficult to determine whether groundwater contamination is more common in this county.

Table 4. Antimicrobial resistance in *Enterococcus* isolates from surface water.

Source	Total Isolates	VAN		Resistant Isolates						GEN	
				CHL		AMP		STR			
		n	%	n	%	n	%	n	%	n	%
Craven	55	4	7	0	0	0	0	0	0	0	0
Wayne	65	0	0	1	1	0	0	5	7	0	0

4. Discussion

This study shows that *E. coli*, enterococci, and *Salmonella* can be found in some surface waters, and low levels of resistance to clinically relevant antibiotics can be found sporadically. *E. coli* and enterococci are present more frequently and at higher levels in surface water than *Salmonella*, but few groundwater sources in our sample were contaminated with any of these organisms. Antibiotic treatment is usually reserved for complicated or invasive disease; in this study sulfamethoxazole, ampicillin, gentamicin, chloramphenicol, ciprofloxacin, and ceftriaxone were chosen as the most clinically relevant antibiotics [20].

Results of this study are in line with previous findings in North Carolina surface and groundwaters. A previous study of eastern N.C. groundwater sources not on or adjacent to CAFOs found that *E. coli* occurrence is relatively uncommon, and when *E. coli* is present the number of organisms is low. Antibiotic resistance in *E. coli* isolates from the groundwater sources examined was also uncommon, but similar to findings in this study, resistance to ampicillin was detected [9]. Another study of eastern N.C. surface and groundwaters near row crop farms was consistent with this study, finding that *E. coli* and enterococci were common in surface waters, but rare in groundwater. *E. coli* and enterococci were found at similar levels to this study, although *Salmonella* was more common in surface waters. As in this study, resistance to ampicillin and sulfamethoxazole was found in *E. coli* from surface waters, but no resistance to ciprofloxacin was found [21].

Analysis of surface and groundwater samples found only low levels of *Salmonella* in surface water, and none in any of the groundwater samples tested. One survey of North Carolina watersheds found *Salmonella* in surface waters, and antibiotic resistant *Salmonella* were most common in a watershed impacted by hog production [22], but *Salmonella* has also been previously found in environmental surface waters unrelated to hog CAFOs [11]. However, groundwater sources may be less vulnerable to contamination even if they are near CAFOs. One study of surface and groundwater samples adjacent to North Carolina hog CAFOs found *Salmonella* in surface waters, including *Salmonella* resistant to ampicillin, chloramphenicol, and sulfa drugs, but no resistance to ceftriaxone or ciprofloxacin. However, no *Salmonella* was detected in groundwater [21]. *Salmonella* contamination of groundwater appears to be uncommon. Bacteria resistant to multiple antibiotics, a growing public health concern, appear to be rare. There are a few limitations of this study. There were a total of 100 samples, with four isolates of each organism taken (if available) from each positive sample, resulting in a small sample size for antimicrobial susceptibility testing. For some samples, organism numbers were low enough that four colonies were not available for isolation. Other studies of N.C. surface and groundwater suggest that antibiotic resistance is present at low levels; if antibiotic resistant organisms are rare, it is possible our sample size underestimates the presence of these organisms.

Resistant bacteria are relatively uncommon in these eastern N.C. surface and groundwaters. However, their presence could pose a risk of human exposure via ingestion of groundwater or primary contact recreation in surface waters. Continued surveillance and identification of potential sources that may release antibiotic resistant enteric bacteria into the environment, whether of human or animal origin, is vital to minimize human exposures to these pathogens via environmental waters.

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Abbreviations

The following abbreviations are used in this manuscript:

CAFO	Concentrated animal feeding operation
MPN	Most probable number

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