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Antimicrobial Resistance in *E. coli* Isolated from Residential Water Wells in South Central Virginia

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Abstract

Approximately 1.7 million Virginians rely on wells, springs or cisterns for water supply and water safety is of particular concern to them. Additionally, due to wide application of antibiotics for human health and agricultural practices, the emergence of antimicrobial resistant (AMR) bacteria and their presence in the environment is a potential health threat. The presence of AMR bacteria in ground water from private water wells hasn't been investigated and the risk of encountering AMR bacteria is unknown. The aim of the present study was to evaluate the prevalence and AMR of *E. coil* in water samples from private wells. Pulsed-field gel electrophoresis (PFGE) profiles of the isolated *E. coli* were further explored for genomic diversity and environmental association to identify potential pathways of the bacterial transmission with regard to the routes of contamination. A total of 823 water well samples from 10 counties in the South Central region of Virginia were obtained between February, 2016 and March, 2017. *E. coli* was detected in 3.7% of total samples.. Approximately 3% of the tested *E. coli* were resistant to at least four antimicrobials. Four isolates were non-susceptible (either resistant or intermediate) to more than seven antimicrobials. No isolates had matching PFGE profiles demonstrating that the isolated *E. coli* strains had a high degree of genomic diversity. This study clearly demonstrates a potential hazard arising from contaminated water wells and emphasizes the importance of regular water quality monitoring for those relying on private water wells.

Keywords: *Water Quality; E. coli; Antimicrobial Resistance; Pulsed-Field Gel Electrophoresis; Genomic Diversity*

Introduction

Over 15 million U.S. households rely on private water wells for drinking water [1] and approximately one of five Virginians (~1.7 million people) relies on wells, springs or cisterns for private water supply [2]. Groundwater contamination, biological and chemical, can originate from many sources, including landfill seepage, failed septic tanks, underground storage tanks, fertilizers and pesticides, and surface water runoff [1,3]. Most biological contamination (bacteria, viruses, and parasites) comes from humans and animal fecal materials [4]. *E. coli*, an inhabitant of the gastrointestinal tracts of warm-blooded animals, is widely used as an "indicator" of the microbiological quality of water [5]. The detection of the bacterial indicator in drinking water suggests the presence of pathogenic organisms that are sources of waterborne diseases [6]. For 2011-2012 in the US, 32 drinking water-associated outbreaks of microorganisms were reported, accounting for at least 431 cases of illness, 102 hospitalizations, and 14 deaths [7]. In 2013, Benjamin., *et al*. [8] reported the presence of high levels of *E. coli* in various water sources, including water wells, on the Central California coast. Water safety should be a concern for those using a private water supply.

The wide application of antibiotics in human and agricultural practices has led to the development and large-scale dissemination of antimicrobial resistant (AMR) bacteria. Their emergence in the environment [9] has become a serious health threat [10-13]. Most of

studies of AMR in *E. coli* have been conducted in clinical settings or in association with farming practices [14-18] where frequent exposure to antimicrobial agents is possible. Several studies found that different species of bacteria from diverse sources (human, animal, environmental) are able to exchange and shuffle genes, including those that confer AMR [9,19,20]. Savard and Perl [21] found that reported rates of multi-drug resistance (MDR) in microorganisms have been increasing and that infections from these microorganisms are no longer limited to hospital or clinical environments. Consequently, the increased prevalence of MDR bacteria in the environment and the potential impact on human and animal health is of world-wide concern [12]. In a previous study [22], we also documented a high prevalence of AMR in *E. coli* isolated from environmental samples, including farm animals, wildlife, and food samples acquired through the Internet and from local retail markets.

The presence of AMR *E. coli* in the surface environment strongly suggests that groundwater may also carry these bacteria. The presence of AMR *E. coli* contamination in food materials, including water, represents not only a possible food safety threat but also a biological threat for AMR transmission [23]. The occurrence of AMR *E. coli* in groundwater is poorly documented in general and, to our knowledge, the prevalence of AMR in and genetic relatedness of *E. coli* isolated from private water wells in South Central Virginia are unknown. The present study investigated the prevalence of AMR *E. coli* isolated from residential water wells in South Central Virginia. Pulsed-field gel electrophoresis (PFGE) profiles of isolated *E. coli* were further explored for their genomic diversity and environmental association to assess potential pathways of the bacterial transmission with regard to the routes of contamination. This study aligns with the World Health Organization's call for improved surveillance of AMR in the environment [24].

Materials and Methods

Water samples tested: A total of 823 samples from residential water wells in 10 counties (Amelia, Chesterfield, Dinwiddie, Goochland, Greenville, Henrico, Powhatan, Prince George, Surry, and Sussex) of the South Central region of Virginia (Figure 1) were collected from January, 2016 to March, 2017. USEPA general sampling procedures for drinking water [1] were followed. Water samples were obtained from various faucet locations (i.e. kitchen, bathroom, well tank, outdoor). In brief, faucets were flushed for at least 10 min prior to sample collection in USEPA-approved 120-ml sterile plastic bottles containing sodium thiosulfate (IDEXX Laboratories, Inc., Westbrook, ME). The containers were sealed immediately after filling and transported on ice or in chilled coolers to a commercial water testing laboratory (Chesterfield, VA). Samples were maintained at 4°C and processed for microbial testing within 24 h using Colilert-24 culture media (IDEXX Laboratories, Inc.). Samples were incubated for 24 h at 35°C and then evaluated. The presence of *E. coli* was indicated by yellow coloration and fluorescence under long-wave UV light at 365 nm [25]. These *E. coli*-positive samples were frozen and transported to our laboratory where they were held at -80°C until used for *E. coli* isolation.

***E. coli* isolation and identification:** *E. coli* isolation and identification were performed following AOAC-approved or performance-tested methods [26]. Samples were thawed in a refrigerator at 4°C after which a loopful of culture from each bottle was streaked on eosine-methylene blue agar (EMB; unless otherwise stated, all media were from Becton, Dickinson and Company, Sparks, MD). Representative purple colonies with or without a green metallic sheen were evaluated by a gram-stain kit (Fisher Diagnostics, Middletown, VA) followed by microscopic examination of colony morphology. Colony identification as *E. coli* was confirmed using the API 20E identification system (bioMerieux, Inc., Durham, NC). Three isolates per water sample were obtained and suspended in tryptic soy broth (TSB) containing 20% glycerol, and stored at -80°C until used to evaluate antimicrobial resistance (AMR) and for pulsed-field gel electrophoresis (PFGE).

Antimicrobial Susceptibility Testing: Following the procedure described by Kim., *et al*. [22], antimicrobial susceptibility tests were performed on Mueller-Hinton agar (MHA) using the Kirby-Bauer disk diffusion method [27]. The isolates were tested for susceptibility to 12 antimicrobials representing 9 different antimicrobial categories (Table 1). Antimicrobial-impregnated disks were positioned on the *E. coli*-inoculated MHA plates that were then incubated at 37°C for 24h. Antimicrobial susceptibility, classified as "resistant", "intermediate" and "susceptible" was interpreted in accordance with criteria established by the National Committee of Clinical Laboratory Standards (NCCLS) [27]. In addition, bacteria classified as either resistant or intermediate were defined as "non-susceptible" and those exhibiting

resistance to at least one antimicrobial agent in three or more antimicrobial categories were defined as MDR [28,29]. *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)		
			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 (CLSI 2015)[a].*

[a]Interpretive criteria: S: Susceptible; I: Intermediate; R: Resistant to Antimicrobial Agents Tested.

Pulsed-field gel electrophoresis (PFGE): PFGE profiles of the *E. coli* isolates were used to evaluate genomic diversity, environmental associations, and strain relatedness. One randomly selected isolate per water sample was used for PFGE evaluation. Following a standard protocol [30], *E. coli* isolates were grown on tryptic soy agar with 5% defibrinated sheep blood at 37°C for 24h. Cells were suspended in Tris-EDTA (TE) buffer (pH 8.0) and adjusted to an optical density of 1.0 to 1.1 using a spectrophotometer (Model G10S UV-Vis, Thermo Scientific, Madison, WI) at 610 nm. An aliquot of each cell suspension was mixed with proteinase K (Fisher Scientific, Fair Lawn, NJ) before being mixed with melted 1.0% Seakem Gold agarose (SKG, Lonza, Rockland, ME) containing sodium dodecyl sulfate in TE buffer. The mixture was dispensed immediately into one plug mold well (Bio-Rad, Hercules, CA). Each solidified plug was transferred to a conical tube containing cell lysis buffer (TE buffer, 1% sarcosyl, and 0.1 mg of proteinase K per ml). Cells were lysed for 30 minutes at 57°C in an incubator with shaking at 100 rpm, the plugs were then washed with ultrapure water (reagent grade type I, EVOQUA Water Technologies, Lowell, MA) and TE buffer. A 2-mm-wide slice was cut from each plug which was then incubated sequentially at 37°C for 5 minutes with 1 X restriction buffer solution (Fisher Scientific), at 37°C for 90 minutes with a restriction enzyme mixture containing 50 U of *Xba*I, and then at 24°C for 5 minutes with 0.5 X Tris-borate-EDTA from a 10 X stock (TBE; Fisher Scientific). The plug slices were anchored to a gel mold comb using freshly made 1% SKG agarose. A plug slice made from *Salmonella* Braenderup (BAA-664, American Type Culture Collection, Manassas, VA) was also loaded onto the gel comb as a marker. Electrophoresis was conducted using a CHEF Mapper system (Bio-Rad) at 6 V/cm and a 120° angle, with an initial switch time of 6.76s and a final switch time of 35.38s. The switch times were set using the auto algorithm function to better separate fragments in the 50- to 400-kb range. Each gel was electrophoresed in 0.5 X TBE for about 18.5h and then stained with SYBR Green I nucleic acid gel stain (Lonza). The band patterns of each stained gel were observed under UV illumination, recorded with an EC3 imaging system with an OptiChemi HR camera (UVP, Upland, CA), and analyzed using VisionWorksLS image acquisition and analysis software (version 6.4.3, UVP).

Data analysis: PFGE band patterns were converted into binary codes that were then evaluated using Exter NTSYSpc software (version 2.2, Applied Biostatistics, Port Jefferson, NY) with unweighted pair group clustering of arithmetic averages to generate a dendrogram of genotypic relatedness.

Results and Discussion

This study investigated the presence of AMR and the genetic relatedness of *E. coli* detected in samples obtained from private water wells in South Central Virginia. *E. coli* was detected in 30 (3.7%) out of 823 samples collected during the study period. *E. coli* was detected in samples collected from six of the ten counties represented. Figure 1 shows locations of *E. coli* positive samples (the associated PFGE band patterns, which will be discussed below, are also enumerated). Of these 30 samples, the prevalence of *E. coli* was the highest in Chesterfield (9, 30%) county, followed by Prince George (8, 33.3%), Dinwiddie (7, 23.3%), Sussex (3, 10%), Henrico (2, 6.7%), and Amelia (1, 3.3%).

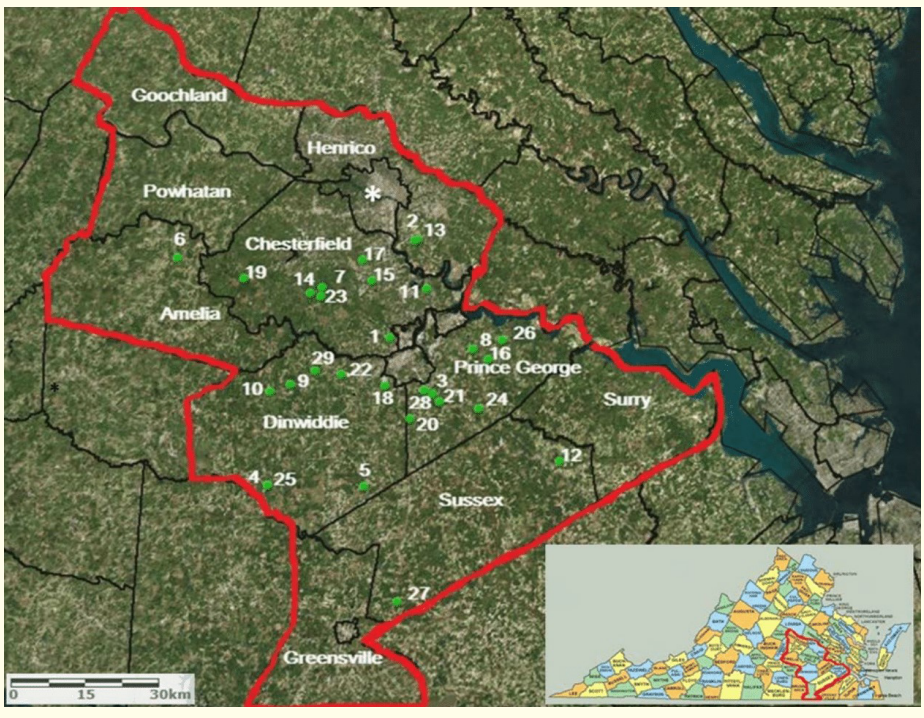

Figure 1: *Map of Virginia (inset, http://www.yoderdairies.com/county-map-virginia.html) showing counties (red-lined area) where water well samples were collected. Sampling sites with E. coli positives (green dots) and corresponding PFGE pattern numbers are indicated. Richmond (*), Virginia's capital city, is indicated as a location reference. Map was generated using a geographic information system software (ArcGIS).*

Ninety isolates from 30 water samples (3 isolates per sample) were confirmed as *E. coli* by the API20E system. Nineteen different API profiles were obtained (data not shown). Of these, a majority of *E. coli* isolates showed either 5144572 (35.6%), 5044572 (20.0%), or 5144552 (15.6%) profiles. One profile (5044552) was found in 5.6% of the isolates whereas fifteen different profiles, representing from 1.1 to 3.3%, were found among the remaining isolates.

The ninety *E. coli* isolates were subjected to antimicrobial susceptibility testing (Table 2). Only 38 (42.2%) isolates were susceptible to (i.e. killed by) all tested antimicrobials, indicating 57.8% of *E. coli* were non-susceptible (either resistant or intermediate) to one or more antimicrobials. Eighteen (20.0%) isolates were resistant to one or more antimicrobials, and three (3.3%) isolates were resistant to four antimicrobials. However, none of the isolates exhibited a resistance to three or more categories of antimicrobials, which is considered as MDR bacteria. The present study also found four isolates that were non-susceptible to more than seven antimicrobials with varying API profiles (5044572, 5144542, 5144552, and 5144572). Three of these isolates were obtained from water samples collected from outside faucets while one was obtained from a bathroom faucet.

Occurrence		**Quantity of antimicrobial agents to which *E. coli* isolates demonstrate resistance or non-susceptibility[c]**							
		1	2	3	4	5	6	7	8
Resistant	Numbers	11	4	0	3	0	0	0	0
	Prevalence (%)	12.2	4.4	0	3.3	0	0	0	0
Non-susceptible (R+I)[b]	Numbers	16	8	11	5	2	6	3	1
	Prevalence (%)	17.8	8.9	12.2	5.6	2.2	6.7	3.3	1.1

Table 2: *Ninety E. coli isolates obtained from 30 water well samples (3 isolates per well) between January of 2016 and March of 2017 that exhibit resistance to one or more antimicrobial agents[a].*

[a]Of ninety isolates, 38 (42.2%) were susceptible to all tested antimicrobial agents; susceptibility categorization was carried out in accordance with interpretive criteria provided by the National Committee of Clinical Laboratory Standards (NCCLS) recommendations (CLSI 2015).

Resistance to STR was the most common in 14 (15.6%) isolates, followed by AMP (5.6%), TOB (5.6%), GEN (3.3%), and TCY (3.3%) (Figure 2). No resistance was found to NAL, CHL, and SXT, yet each showed intermediate resistance of 13.3, 5.6, and 1.1%, respectively. The most effective antimicrobials tested in the present study were MEM and CIP showing 100% susceptibility. In other words, all 90 *E. coli* isolates were susceptible only to MEM and CIP suggesting that these antimicrobials may be still effective for treatment of *E. coli*, which is an opportunistic human pathogen. In a study of the prevalence of AMR in *E. coli* isolates obtained from animal and food samples, Kim., *et al*. [22] also found an overall susceptibility to MEM and CIP and similar pattern of resistance to the rest of antimicrobials.

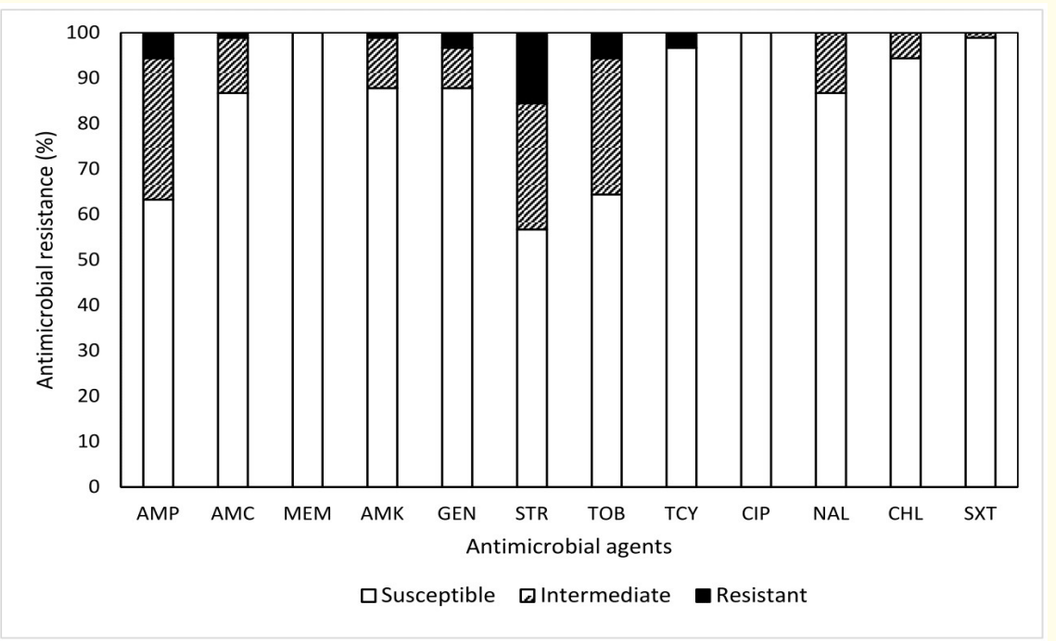

Figure 2: *Prevalence of resistance to 12 antimicrobial agents in 90 E. coli isolates obtained from 30 water wells (3 isolates per well).*

Although the prevalence of AMR in *E. coli* isolated from private wells in the U.S., especially in Virginia, are unknown to our knowledge, several studies [31-35] reported the prevalence of AMR in *E. coli* isolates obtained from surface and water well samples from outside of the U.S. Mukhopadhyay., *et al*. [33] evaluated the microbial quality of drinking water from water wells in urban and rural India. They reported that although the majority of coliforms isolated from water wells were susceptible to commonly used antimicrobials, a significant number of isolates were resistant to AMP (76.5%), which was much higher than our findings (5.6%). However, resistance to GEN was 2.5% in their study, which is in reasonable agreement the result (3.3%) from the present study. Jongman and Korsten [32] reported that *E. coli* isolates from river water in South Africa were resistant to AMP (8.6%), AMC (2.9%), CHL (11%), STR (5.7%), TCY (11%), and SXT (11.4%). Ram., *et al*. [34] also investigated AMR in *E. coli* isolates from surface (river) water in India. They reported that 28% of the isolates were susceptible to tested antimicrobials. However, 20% were resistant to AMP and TCY. Amaya., *et al*. [31] reported that 9.2% of well water samples tested in León, Nicaragua, carried AMR in *E. coli* and among them, 19% were resistant to AMP, CHL, GEN, NAL, and SXT. From the studies above, MDR in *E. coli* varied from 1.1 to 21%. With regards to the discrepancy among studies, Doyle., *et al*. [36] indicated that 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. Kim., *et al*. [22] cited that it is also possible for *E. coli* isolates to develop different degrees of resistance to antimicrobials depending upon the type of environment to which they have been exposed and their genetic background.

A total of 29 distinct PFGE profiles (one sample did not produce distinct band pattern) demonstrating ≥55% genetic similarity were obtained among 30 *E. coli* isolates of water samples (Figure 3). Within the overall distinct band patterns, seven differentiable patterns were obtained from Dinwiddie County and eight from Chesterfield and Prince George, respectively. The PFGE patterns (patterns -6, -7, -18, -23, and -24) of the isolates with high prevalence of non-susceptibility to six or more antimicrobials displayed at least 57% similarity. Two profiles, pattern-17 from Chesterfield County and Pattern-18 from Dinwiddie County, displayed 79% similarity. The highest similarity of PFGE profiles between isolates from the same counties was 71% and they were patterns -14 and -15 obtained from Chesterfield County. Even the profiles (patterns -9 and -10) of strains isolated from sampling sites in very close proximity (less than 100m) displayed relatively lower similarity (approximately 63%) than what otherwise might be expected.

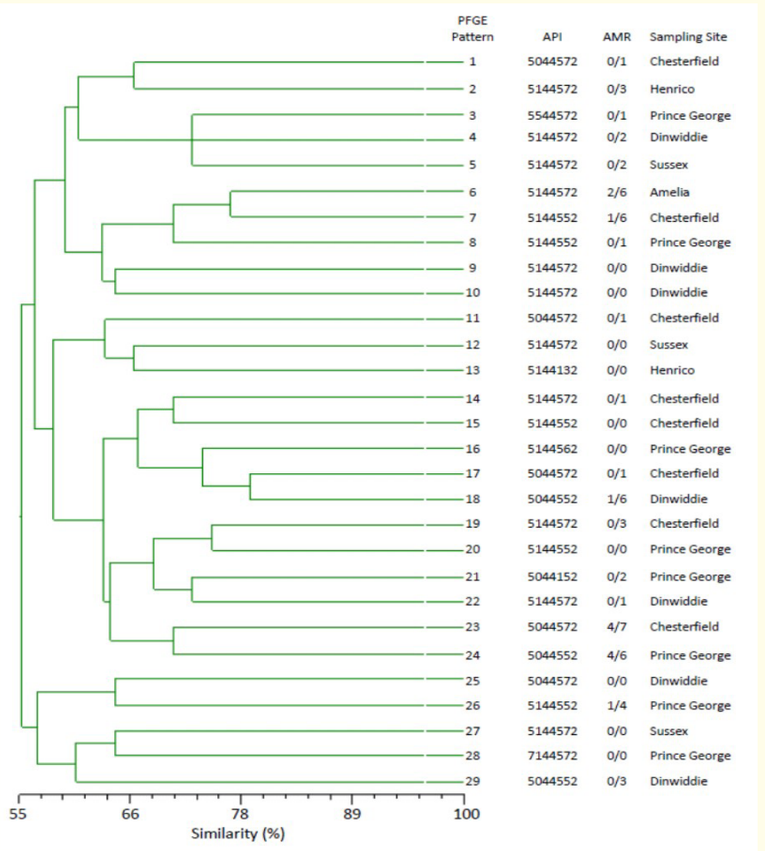

Figure 3: *Dendrogram of XbaI pulsed-field gel electrophoresis profiles of 29 E. coli isolates from water wells; AMR, number of antimicrobials that isolates show resistant/non-susceptible (resistant and intermediate); sampling site is shown in county.*

Findings from the present study were consistent with those of Kim., *et al.* [22] on animal and food samples. None of the PFGE profiles of *E. coli* isolates from that study demonstrated any correlation with their prevalence to antimicrobials. In contrast, Zhao., *et al*. [37] showed a good correlation of bacterial PFGE profiles with their AMR profiles. This discrepancy may be due to the difference in types of microorganisms and antimicrobial panels used or to the type of restriction enzyme. Regardless of the discrepancy, the results from the present study and our prior study [22], though from a limited geographical region, show a level of consistency between the prevalence of resistance to certain antimicrobial categories evaluated and their usage levels in the US from 2000 and 2010 [30] showing the highest prevalence of resistance to antimicrobial categories of penicillins, β-lactams, and aminoglycosides.

Conclusion

In conclusion, findings from the present study confirm the high prevalence of non-susceptible E. coli to broad-spectrum antimicrobials in water wells. This study shows that bacterial contamination of water wells represents a potential health hazard due not only to the presence of potentially pathogenic organisms but also that treatment of bacterial diseases arising from drinking contaminated water may be complicated by the presence of AMR bacteria. Working with local watershed committees to better understand watershed areas and promoting stewardship of these waterways can improve water quality for homeowners and farms dependent upon private water well supply. Efforts to inform and educate private well owners about the importance of a regularly scheduled program of water quality monitoring and preventative well maintenance are encouraged. Continued research efforts on a larger scale are underway to determine the cause(s) of the observed prevalence of AMR in bacteria in relation to the environment and their genomic relatedness. Our findings may benefit the National Antimicrobial Resistance Monitoring System by providing data of AMR surveillance in water wells, further assisting in the development of new antimicrobials and promoting interventions to reduce resistance among foodborne bacteria.

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Disclaimer

Findings in this study simply indicate the occurrence of *E. coli* in water wells by the request of those who were interested in their water quality and, therefore, may not be representative of all water wells in the study area. The incidence of prevalence in each county may be higher than the actual quality of water in wells.

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