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Occurrence of vancomycin-resistant and -susceptible *Enterococcus* spp. in reclaimed water used for spray irrigation

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ABSTRACT

Reclaiming municipal wastewater for agricultural, environmental, and industrial purposes is increasing in the United States to combat dwindling freshwater supplies. However, there is a lack of data regarding the microbial quality of reclaimed water. In particular, no previous studies have evaluated the occurrence of vancomycin-resistant enterococci (VRE) in reclaimed water used at spray irrigation sites in the United States. To address this knowledge gap, we investigated the occurrence, concentration, and antimicrobial resistance patterns of VRE and vancomycin-susceptible enterococci at three U.S. spray irrigation sites that use reclaimed water. We collected 48 reclaimed water samples from one Mid-Atlantic and two Midwest spray irrigation sites, as well as their respective wastewater treatment plants, in 2009 and 2010. Samples were analyzed for total enterococci and VRE using standard membrane filtration. Isolates were purified and then confirmed using biochemical tests and PCR. Antimicrobial susceptibility testing was conducted using the Sensititre® microbroth dilution system. Data were analyzed by two-sample proportion tests and one-way analysis of variance. We detected total enterococci and VRE in 71% (34/48) and 4% (2/48) of reclaimed water samples, respectively. *Enterococcus faecalis* was the most common species identified. At the Mid-Atlantic spray irrigation site, UV radiation decreased total enterococci to undetectable levels; however, subsequent storage in an open-air pond at this site resulted in increased concentrations of enterococci. *E. faecalis* isolates recovered from the Mid-Atlantic spray irrigation site expressed intrinsic resistance to quinupristin/dalfopristin; however, non-*E. faecalis* isolates expressed resistance to quinupristin/dalfopristin (52% of isolates), vancomycin (4%), tetracycline (13%), penicillin (4%) and ciprofloxacin (17%). Our findings show that VRE are present in low numbers in reclaimed water at point-of-use at the sampled spray irrigation sites; however, resistance to other antimicrobial classes is more prevalent, particularly among non-*E. faecalis* isolates.

1. Introduction

As the world population increases and global water use escalates, freshwater resources continue to dwindle. To alleviate pressures on freshwater resources, countries—including the United States—are reclaiming treated municipal wastewater for potable and nonpotable reuse (EPA, 2012). This reclaimed water has been defined as "municipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for a range of purposes"(EPA, 2012). In the United States, reclaimed water is used in landscape irrigation, food crop irrigation, snowmaking, groundwater recharge, power production, and indirect and direct potable reuse (EPA, 2012). With increasing reclaimed water use, the potential public health impacts due to microbial contamination of reclaimed water need to be explored and addressed.

Previous studies have shown that a number of bacterial pathogens can survive wastewater treatment including methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and enterococci (Levantesi et al., 2010; Nagulapally et al., 2009; Rosenberg Goldstein et al., 2012, 2014). Vancomycin-resistant Enterococci (VRE), in particular, have recently been isolated from wastewater effluent (Garcia et al., 2007; Nagulapally et al., 2009; Rosenberg Goldstein et al., 2014) and could persist in distribution systems that supply reclaimed water to spray irrigation sites.

VRE are gram-positive, opportunistic human pathogens that are resistant to vancomycin (a drug of last resort) and can cause

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urinary tract infections, wound infections, bacteremia and endocarditis (CDC, 2009). Between 2006 and 2007, *Enterococcus* spp. was the third most commonly reported pathogen causing healthcare-acquired infections in the United States (Hidron et al., 2008). Twelve percent and 4% of pathogens recovered from healthcare-acquired infections were *Enterococcus* spp. and VRE, respectively (Hidron et al., 2008). And by 2010, *Enterococcus* spp. became the second leading cause of healthcare-acquired infections (Sievert et al., 2013). Enterococci, in general, are tolerant to an array of environmental stressors, including extreme temperatures (5–65 °C), variable pH levels (4.5–10), and high NaCl concentrations (Fisher and Phillips, 2009). Due to the higher tolerance of enterococci to chlorination, these microorganisms can withstand wastewater treatment processes—including tertiary treatments involving chlorination—and persist in the environment (Castillo-Rojas et al., 2013; Varela et al., 2013).

Hospital effluent discharged to municipal wastewater treatment plants has been identified as an important initial source of environmental contamination of VRE (Varela et al., 2013). Antibiotic-resistant enterococci has been recovered from treated municipal wastewater effluent in the United States, China, and Portugal (Ferreira da Silva et al., 2006; Garcia et al., 2007; Huang et al., 2012; Martins da Costa et al., 2006), and VRE, specifically, has been isolated from treated wastewater effluent in the United States and the United Kingdom (Beier et al., 2008; Caplin et al., 2008; Rosenberg Goldstein et al., 2014).

However, to our knowledge, there are no published studies analyzing reclaimed water recovered from U.S. spray irrigation sites (at point-of-use) for the presence of VRE and total enterococci. In this study, we evaluated the occurrence, concentration, and antimicrobial susceptibilities of VRE and total enterococci recovered from reclaimed water used at three U.S. spray irrigation sites. We also evaluated the impact of storing reclaimed water in open-air ponds on levels of VRE and total enterococci.

2. Materials and methods

2.1. Study sites

We sampled three spray irrigation sites that use reclaimed water: one Mid-Atlantic site and two Midwest sites. All sites were chosen based on the willingness of the site operator to participate.

The Mid-Atlantic spray irrigation site (Mid-Atlantic SI1) receives wastewater effluent from a tertiary wastewater treatment plant (WWTP) in an urban area that has been described previously as Mid-Atlantic WWTP1 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (681,390 m^3/day) at this plant is comprised of domestic and hospital wastewater and the plant employs the following treatment steps: screens, primary clarifier, primary aeration tank, secondary aeration tank, secondary clarifier, multimedia filter, chlorination, dechlorination and discharge. The chlorination dose at this plant was 2–3 mg/L, followed by dechlorination with sodium bisulfite such that the chlorine residual in effluent is <0.1 mg/L. This treated effluent is then piped to Mid-Atlantic SI1. Once it arrives at Mid-Atlantic SI1 the effluent passes through a double-walled aluminum screen and is then treated with 254 nm wavelength ultraviolet (UV) radiation bulbs that produce a minimum of 30,000 microwatt seconds per square centimeter. After UV treatment, the water is pumped into an open-air storage pond at a rate of 230,000 gallons per day with a peak capacity of 4 million gallons. The reclaimed water is then pumped from the storage pond to spray irrigation heads for use in landscaping (Fig. 1).

Midwest spray irrigation site 1 (Midwest SI1) receives wastewater effluent from a tertiary WWTP in a rural area that has been described previously as Midwest WWTP1 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (1363 m^3/day) at this plant is comprised of domestic wastewater and agriculturally influenced stormwater, and the plant employs the following treatment steps: screens, activated sludge lagoons, clarifiers, seasonal chlorination (and dechlorination), and discharge. Seasonal chlorination occurs at this plant in June, July, and August, and during these times the chlorination dose is 4 mg/L with a contact time to assure a chlorine residual of 0 mg/L in effluent. The effluent is then piped to Midwest SI1 where it undergoes no additional treatment, is stored in an open-air storage pond and is then pumped to spray irrigation heads for use in landscaping (Fig. 1).

Midwest spray irrigation site 2 (Midwest SI2) receives wastewater effluent from a tertiary WWTP in a rural area that has been described previously as Midwest WWTP2 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (1439 m^3/day) at this plant is comprised of domestic wastewater, wastewater from a food production facility, and agriculturally influenced stormwater, and the plant employs the following treatment steps: screens, sequencing batch reactor, lagoon cell A, lagoon cell B, lagoon cell C, lagoon cell D, lagoon cell E, and discharge. The unchlorinated effluent from this plant is piped to Midwest SI2 where it undergoes no additional treatment, is stored in an open-air storage pond and is then pumped to spray irrigation heads for use in landscaping and crop irrigation (Fig. 1).

All of the treatment processes described above at the wastewater treatment plants and spray irrigation sites are still in effect as of 2016. In addition, the population and land use dynamics that are relevant to each treatment plant have not changed, and therefore, it is unlikely that the quality and substance of raw wastewater influent entering the studied plants has changed substantially between the time that samples were collected and the publishing of this study.

2.2. Sample collection

A total of 48 reclaimed water samples were included in this study (Table 1). All samples were collected between August 2009 and October 2010 during multiple visits to each site. The timing of sample collection was determined by the site operators. Fig. 1 indicates the specific locations where the samples were collected. All samples were collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles and transported to the laboratory at 4 °C.

2.3. Isolation

Standard membrane filtration was used to isolate total enterococci and VRE from the reclaimed water samples (EPA, 2002). Ten-fold dilutions of each sample were filtered through 0.45 μm, 47 mm mixed cellulose ester filters (Millipore, Billerica, MA). Filters were then plated in duplicate on membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) agar (EMD Millipore, Billerica, MA) to isolate total enterococci, and mEI agar modified with 16 μg/mL of vancomycin to isolate VRE. Plates were incubated at 41 °C for 24 h. Colonies with blue halos were considered presumptive total enterococci and VRE. These colonies were purified on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and archived in Brucella broth (Becton, Dickinson and Company) with 15% glycerol at −80 °C. *E. faecalis* ATCC 29,212 was used as a positive control and phosphate buffered saline was used as a negative control throughout the isolation process.

2.4. Identification

Total enterococci and VRE were confirmed and identified using the Gram stain, the catalase test, detection of pyrrolidonyl